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POSSIBILITIES OF DRIED BLOOD SPOTS AS A MATRIX IN THERAPEUTIC DRUG MONITORING OF ANTIEPILEPTIC DRUGS IN CHILDREN

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Possibilities of dried blood spots as a matrix in therapeutic drug monitoring of antiepileptic drugs in children
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ABSTRACT

For children with epilepsy, therapeutic drug monitoring (TDM) of antiepileptic drugs (AEDs) is essential for treatment. TDM monitoring requires two visits to a health care unit, first for collection of the blood sample before the morning dose and a few days later the family will travel once again to see the pediatrician. For this patient group, blood from a fingerprick, collected on a filter paper at home and sent by mail to the laboratory as a dried blood spot (DBS), could be beneficial and facilitate everyday life.

The aim of this thesis has been to investigate whether it is possible to use DBS as a matrix for TDM of common AEDs interchangeably with plasma. A second aim was to study the DBS self-sampling process, to see if guardians of children with epilepsy were able to collect DBS samples with sufficient quality and find what factors influenced a successful collection.

Precise and robust liquid chromatography tandem mass spectrometry (LC-MS/MS) methods, using DBS as a matrix, were developed and validated for four major AEDs. The methods met acceptance criteria from the European Medicine's Agency (Paper I and III). Patient samples were collected from children treated with carbamazepine (CBZ), lamotrigine (LTG), levetiracetam (LEV) and valproic acid (VPA) to perform bridging studies and compare DBS concentrations with plasma.

Results concluded that a factor was needed to convert DBS concentrations for CBZ and VPA, to estimated plasma concentrations (Paper II). In the clinical evaluation, only 4 out of 190 comparisons of DBS and plasma concentrations resulted in different dose recommendations The DBS self-collection process was evaluated by comparing the quality of samples obtained from a group of pediatric nurses with a group of guardians to children with epilepsy. Results showed that guardians could collect acceptable quality DBS samples from their children (Paper IV).

A mixed method study showed that most of the guardians (80%) found self-sampling at home desirable after a training session. Factors for successful self-sampling were; high motivation, prepared guardians, flexible education with support from a nurse, effective communication between the guardian and the child and that guardians were willing to take on the role as performers (PaperV).

The conclusion was that children with epilepsy and their guardians can use DBS as an alternative matrix for self-sampling, and that DBS concentrations of CBZ, LTG and VPA can be analyzed for TDM purposes. DBS for LEV can be collected for compliance queries or for patients where the alternative is no sample at all. These patients are monitored closely and in cases where DBS samples collected at home result in a concentration deviating from the expected or at risk of adverse effects, it is recommended to also collect a plasma sample.

Guardians of children with epilepsy are interested in performing self-sampling and by being offered DBS self-collection at home these families can save time and have lower stress levels.

LIST OF SCIENTIFIC PAPERS

I. **Linder C**, Andersson M, Wide K, Beck O, Pohanka A. A LC-MS/MS method for therapeutic drug monitoring of carbamazepine, lamotrigine and valproic acid in DBS. Bioanalysis. 2015; 7(16): 2031-39.

II. **Linder C**, Wide K, Walander M, Beck O, Gustafsson LL, Pohanka A. Comparison between dried blood spot and plasma sampling for therapeutic drug monitoring of antiepileptic drugs in children with epilepsy: A step towards home sampling. Clin Biochem. 2016; 50: 412–8.

III. **Linder C**, Hansson A, Sadek S, Gustafsson LL, Pohanka A. Carbamazepine, lamotrigine, levetiracetam and valproic acid in dried blood spots with liquid chromatography tandem mass spectrometry; method development and validation. J Chromatogr B. 2018; 1072: 116–22.

IV. **Linder C**, Neideman M, Wide K, von Euler M, Gustafsson LL, Pohanka A. Dried blood spot self-sampling by guardians of children with epilepsy is feasible: comparison with plasma for multiple antiepileptic drugs. E-publication ahead of print. Therapeutic Drug Monitoring. 2019; Feb 22

V. **Linder C**, Neideman M, Gambell Barroso M, Gustafsson LL, Wide K, Pohanka A, Bastholm-Rahmner P. Guardians' perspective of dried blood spot self-sampling from children with epilepsy in Sweden: a mixed method study. *In manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AED	antiepileptic drug
CBZ	carbamazepine
CEDIA	cloned enzyme donor immunoassay
DBS	dried blood spot
EMA	European Medicines Agency
ESI	electro spray ionization
f_{BC}	fraction in red blood cells
FDA	United States Food and Drug Administration
GC-MS	gas chromatography mass spectrometry
Hct	hematocrit
HEIA	homogenous enzyme immunoassay
HPLC-UV	high performance liquid chromatography-ultra violet detection
LC	liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
LEV	levetiracetam
LLOQ	lower limit of quantification
LTG	lamotrigine
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
QC	quality control
RCPA	The Royal College of Pathologists
SR	Stockholm Health Care Region
TDM	therapeutic drug monitoring
VPA	valproic acid

1 PREFACE

The main focus of this thesis has been to explore and investigate the possibility to use blood collected on filter paper or dried blood spot (DBS) as a matrix for quantification of antiepileptic drugs (AEDs), Figure 1. For patients with epilepsy, treatment with AEDs and in relevant cases therapeutic drug monitoring (TDM) with individual doses are essential for effective treatment. DBS is a matrix that has the potential to be utilized at home for capillary blood self-sampling, and it can facilitate the everyday life for children with epilepsy and their families.

Figure 1. Blood spot collection on a filter paper, DBS
Photo, Johanna Linder.

Method development and validation for DBS has been the main objective in paper I and III. Adaption to the routine laboratory by developing rapid, simple and robust methods with sample traceability has been a main goal.

Bridging studies between measured concentrations in capillary DBS and capillary plasma, was the main objective in paper II and IV. Guardians (parents or someone responsible for the child) collected DBS samples from their children with the help of an instruction video[1] and support from a nurse.

In paper IV and V the patient perspective was investigated. Could guardians collect DBS from their children with acceptable quality? What did they experience during the collection process and what opinions did they have to self-sampling at home?

From my own professional perspective as a clinical biomedical scientist, the quality in all steps from sample collection to the final delivered result, is an important aspect in laboratory analysis. This comprehends good repeatability, credibility and trustworthiness. Also my earlier professional experience as a teacher, with knowledge on the importance of the presentation of information and education, has influenced this work. The presentation of the work in this thesis is discussed from three different perspectives. The *laboratory* perspective, the *clinical* perspective -and the *patient* perspective.

2 INTRODUCTION

2.1 DEVELOPMENT OF DRIED BLOOD SPOT SAMPLING: A HISTORICAL PERSPECTIVE

Blood is collected for a diverse range of investigations and analyses to check physiological and biochemical states related to health status or treatment. The golden standard for blood collection is whole blood collected as fresh venous blood in collection tubes, with the option for further processing of plasma or serum when needed.

The filter paper matrix for the collection of blood samples, has been and is still of high relevance, due to many advantages and its diverse possible applications. Good sample stability over a wide temperature range and collection by non-professional health care workers, made collection possible in developing countries and regions far from hospitals. DBS sampling has been used for a diverse range of purposes as for example newborn screening for metabolic disease, human immunodeficiency virus (HIV) surveillance, clinical chemistry, forensic science, toxicology, biomarker development, drug discovery, human epidemiological and environmental population studies[2].

By the end of the 19th century, the Norwegian physician and scientist *Ivar Christian Bang* (1869-1918) experimented with micromethods for the sampling of blood glucose with filter paper-based matrix in his laboratory[3] in Uppsala and Lund, Sweden. Already then, he criticized methods that required large amounts of blood, by stating: "*Certainly we know many methods for studying the blood in many directions, and our knowledge of blood is still a terra incognita. For determining blood constituents if one could provide methods which require no more blood than can be drawn from the tip of a finger, or the ear vein of a rabbit, it would become possible to explore an inconceivable number of the finer processes of the blood and the cells, with results which a priori are unpredictable*." [3]

In *1963 Dr. Guthrie* with his assistant *A. Susi* worked at Buffalo Children's Hospital in Buffalo, New York and was specifically interested in the diseases of mentally disabled children. They invented a method where blood on filter paper was used to measure phenylalanine levels for detecting phenylketonuria in newborns[4]. Since then, screening of genetic metabolic disorders in newborns has been done throughout the world, collecting drops of blood onto a filter paper by heel or finger stick[5,6].

Historically, DBS methods were qualitative, until the development of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)[7] The improved sensitivity with the LC-MS/MS platform made small amounts of blood in the microliter ranges enough for analysis. One of the first methods based on LC-MS/MS was developed in 1990 for acylcarnitine profiling[8]. Therapeutic drug monitoring (TDM), meaning analysis and evaluation of drug concentrations for the optimization of individual patient drug treatment, has been one of the main areas where DBS methods have been developed[2]. A method for measuring concentrations of antiretroviral drugs in TDM was developed in 2005[9]. Since then, published DBS methods for different drugs and TDM have been steadily increasing.

The history of using DBS at the Clinical pharmacology laboratory, Karolinska University Hospital, Huddinge, started in 1985 when the tropical clinical pharmacology research group developed and used filter paper methods for pharmacokinetic and clinical field studies of chloroquine using high performance liquid chromatography (HPLC-UV) methods[10]. Later other DBS methods for assay of field studies were developed[11–13] .

DBS as an alternative matrix for TDM is promising and desirable since it may be possible for patients to sample themselves at home and send the DBS sample by mail for analysis at the TDM laboratory[14]. This may shift the balance in health care by patient involvement and increased patient autonomy[15,16].

2.2 PROPERTIES OF DRIED BLOOD SPOTS (DBS)

There are many advantages associated with the use of DBS as a matrix, seen from all perspectives, laboratory, clinical, patient and society. One advantage is the minute amount of blood sampled compared to venous sampling. For pharmacokinetic studies in animals the numbers of animals required could be reduced by ~ 60% if DBS were used as matrix replacing traditional blood collection in sample tubes[14], which would contribute to more ethical animal testing[17]. For neonatal care and blood collection the volume of blood collected is particularly important. For a premature newborn baby, the volume of blood in a normal blood test corresponds to as much as 0.3 to 1.3% of their total blood volume, while corresponding roughly to 0.01% of the total blood for an adult. Only 20-50 µL are required for a DBS, compared to 0.5 mL for a micrrotube or 5 mL for collection in a traditional venous tube.

From a patient perspective, capillary sampling is less invasive which explains why it is preferred by many patients[18–20]. DBS sampling can also be performed at home by the patient or a non-professional carer with increased autonomy and reduced costs associated with transport, personnel and material[15]. Self-sampling at home is suitable for clinical and large-scale studies requiring multiple sampling, since it becomes easier and more time efficient for participants[21].

From a laboratory perspective there are advantages when using DBS as matrix instead of plasma or whole blood. It is a dry and less infectious matrix for transportation and handling as compared to blood or plasma[22–24]. DBS cards can usually be transported and stored at room temperature with no requirements of energy-consuming cooling-packages, refrigerators or freezers. Transportation costs, carbon dioxide and waste products can be reduced [24]. Calculations have shown that as much as 100 000 € could be saved during the shipping process in a clinical trial by sending samples as DBS instead of plasma samples that have to be transported on dry ice[21]. In most cases, DBS samples can be stored for longer periods of time with little or no degradation of the drugs[7].

From a societal perspective, DBS sampling has the potential to better meet the United Nations sustainable development goals[25] with reduced transportation, energy savings, less waste materials and equal access to health care.

2.2.1 Whole blood versus plasma

The drug concentration of AEDs in TDM is commonly measured in plasma[26]. Sample collection for TDM purposes is routinely performed as venous sampling (capillary in children) by nurses at the hospital or at an out-patient clinic. The main arguments for measuring in plasma have traditionally been; ease of storage, suitability for chemical analysis and homogeneity of plasma compared to clotted blood. Physiological considerations have been the main argument for measuring drug concentration in whole blood[27,28]. Evaluation of the variability of unbound fraction and the blood-to-plasma concentration (see section 2.4.2) is recommended before deciding whether to use plasma or whole blood for analysis of a specific drug[27].

2.2.2 Composition of blood

Whole blood consists of approximately 45% blood cells and 55% plasma. The hematocrit (Hct), see section 4.3.4, is a measure of volume of the erythrocytes and varies with sex, age and health status. The red blood cells or erythrocytes are the main part of the blood cells and have a diameter of 6-8 µm, a thickness of 2-3 µm and a biconcave disc shape that can tolerate deformation which is necessary for passing through the capillaries[29,30].

Leukocytes and thrombocytes make up approximately 1% of the blood volume. When blood is centrifuged, the erythrocytes are packed at the bottom and the plasma at the top with a buffy coat of leukocytes and thrombocytes in the middle. Plasma contains water, ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^-), energy substrates (glucose, fatty acids, ketone bodies, amino acids), metabolites (ammonia, urea, creatinine, bilirubin), proteins, lipoproteins, hormones and clotting factors. The most abundant proteins are albumins, globulins and fibrinogen[30].

Capillary blood is a mixture of venous and arterial blood and measures for pH, glucose, and blood gases are somewhere in between these two[31]. A fingerprick collection of capillary blood, is probably closer to arterial blood than venous blood, due to the relatively higher pressure on the arterial side[32]. Comparison of endogenous substances between venous and capillary blood have shown differences of less than 5%[33]. Differences between capillary and venous drug concentrations can be seen shortly after drug administration but are unlikely if samples are taken as trough concentrations (just before the morning dose)[27]. Capillary sampling can be performed by non-professionals but the sampling technique can affect the result, e.g. heavy pressure by squeezing the finger might increase the interstitial fluid in the sample, causing dilution. Collectors are instructed to wipe away the first drop of blood since this drop does most likely contain excess interstitial fluid[34]. Calibrators and quality controls (QC) samples for bioanalytical methods are usually prepared using venous blood taken in collection tubes containing anticoagulant, and thus differ from the patient capillary blood which is absorbed directly on filter paper.

2.2.3 The filter paper

The filter paper used for collection of DBS is composed of a structured network of fibers made of polysaccharide cellulose with absorbent ability. The blood components, including the drugs, are bound by and incorporated in the polymers of the filter paper through hydrogen bonds when the paper dries. Small molecule drugs are usually stable in this dry matrix for long periods of time, typically up to several months depending on the drug and paper[14].

For TDM, untreated papers like Whatman 903 Protein Saver Card (GE Health care Ltd, Cardiff, UK) and Perkin Elmer 226 (formerly Ahlström 226, Perkin Elmer, Waltham,USA), have most often been used and both approved by the US Federal Drug Administration (FDA) as collection devices[35,36]. The papers are produced with strict quality control, following ISO9001 manufacturing standards. The choice of filter paper for patient sample collection in TDM analysis needs to be thoroughly considered. Factors that can influence the choice include method performance, price, commercial availability, traceability and adaptability to the laboratory workflow[37]. The work presented in this thesis was developed using Whatman 903 paper. Apart from being quality assured according to clinical laboratory standards institute (CLSI) requirements for mean serum uptake, mean blood absorption time and mean spot diameter by the manufacturer, the filter paper has also been thoroughly tested for variation in analytical results within- and between batches[38].

The dry matrix can increase stability of small drug molecules compared to blood or plasma in collection tubes. Increased stability has been shown for pro-drugs[39], glucuronide metabolites and photosensitive drugs[40,41]. AEDs such as carbamazepine (CBZ), lamotrigine (LTG) and levetiracetam (VPA) have been found to be stable for at least 40 days at room temperature[42].

Blood tends to spread in a heterogeneous manner on the filter paper. This is a chromatographic effect, sometimes described as the `volcano´ effect[43]. Another effect describes the accumulation of solid particles at the outer edges of the bloodspot, causing a rim pattern, called the coffee stain effect[44]. Two processes have been described regarding spotting and spreading of blood on Whatman 903; how blood spreads on top of the substrate and how it spreads horizontal within the paper[45]. Comparisons have been made between different filter papers and the homogeneity of Whatman 903 paper was acceptable using autoradiography for visualization[43]. It was noted, however, that higher concentrations were measured in the edges of the spot for several investigated filter papers[35,46,47]. The chromatographic effect seems to be higher in blood with low Hct[47,48]. Variations in the homogeneity of DBS samples and the relationship between the chromatographic effect and Hct require thorough validation[7,49].

2.2.4 Effects of different hematocrit (Hct) and drop volume

Blood with high Hct has higher viscosity which creates smaller spots on most filter papers and conversely a sample with low Hct spreads more on the paper, Figure 2. If a partial punch is taken from the DBS, a positive or negative bias on measured concentration will correlate to the Hct of the sample[46,47,50–52]. The effect of different Hct values has been identified as the

most influential factor for quantitative DBS sampling methods with more than 15% bias in accuracy at extreme Hcts[49,53]. Hct effects can vary both with concentration and the type of analyte[39,47]. For some drugs, it has been reported that samples with high Hct values, >55%, had reduced recoveries[54,55].

Figure 2. Blood on filter paper with the same pipetted volume but different Hct from 0.25 L/L -0.60 L/L. Blood with low Hct and lower viscosity spreads more than blood with a high Hct.

Hct values in children (6 months to 18 years) can vary between 0.30 to 0.49 L/L[56]. Development and validation of a bioanalytical method needs to at least cover the range of normal Hct values, 0.30-0.50 L/L[50,57].

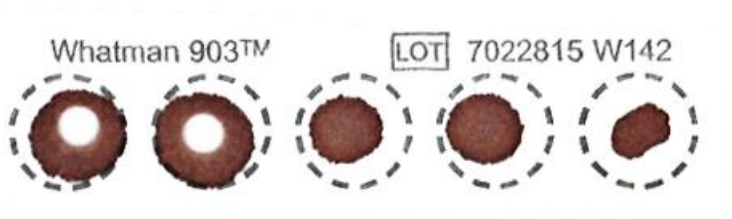


Figure 3. A typical DBS sample from a child collected by the guardian. Collected blood drops have different volumes. The drop to the right is too small and irregular and can give rise to a negative bias due to the small volume.

A filled circle in a Whatman 903 Protein Saver Card contains approximately 50 µL of blood. Blood drops with different volumes are expected during patient sample collection, Figure 3. The spread of the drop of blood on the paper can be affected by various factors. One effect of this is that a small drop usually has a negative bias and large drops have positive bias on the concentration (although within ±10 % for relevant volumes)[35]. Blood drops with a very low volume also have a risk of not saturating the paper[58]. Despite of these possible contributing factors, the spread of blood on a Whatman 903 card has been shown to be linear and the area of the spot corresponds to a specific volume of blood that can be calculated[59], Figure 4.

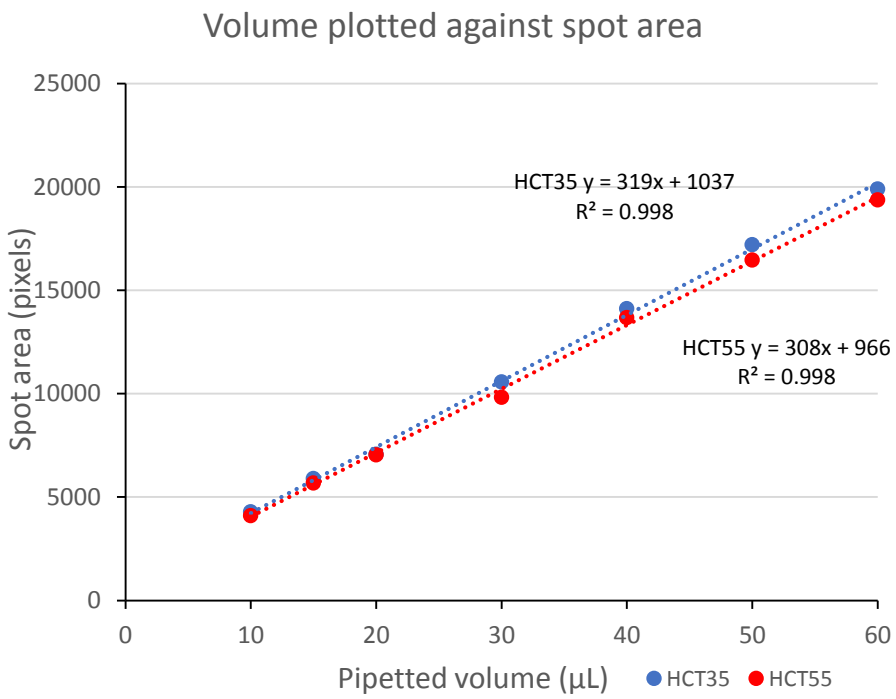


Figure 4. Blood pipetted at different volumes (n=3 for each volume) on a Whatman 903 paper. Area was measured by scanning the blood spots. There is a clear linear relationship between area (pixels) and volume. The area is somewhat affected by the hematocrit.

Since drop volume and Hct affect the measured drug concentration when using DBS, accuracy and precision has to be measured in the validation process over the volume and Hct range expected to be present in the patient group to be studied. An important consideration in method development is the choice of Hct and volume for drops in the production of calibrators and QCs since they will function as a reference value[35].

In order to overcome the problems of unknown volumes and Hct effects, several solutions are now available in the form of automated devices, which can produce a spot with a defined blood volume. The whole spot can be analyzed instead of a partial punch and thereby the Hct effects are limited[55,60–63].

2.3 BIOANALYTICAL METHODS FOR QUANTIFICATION

2.3.1 Immunochemical methods and LC-MS/MS methods

The routine TDM laboratory relies on immunochemical methods for measuring the concentration of CBZ, LTG, LEV and VPA in plasma. These methods include, Cloned Enzyme Donor Immunoassay (CEDIA)[64,65], Homogeneous Enzyme Immunoassay (HEIA),[66] and Quantitative Microsphere System (QMS)[67]. The assays differ in detection technique, but they are based on the principle of antibodies and conjugated drug. The drug molecules in a patient sample compete with the conjugated drug to occupy the free antibodies. Binding of the specific drug of interest is proportional or inversely proportional to the amount of drug present in the sample and is measured through absorbance change either by enzyme activity or turbidimetry (agglutination). Although immunochemical methods are highly sensitive,

they suffer from cross- reactivity issues that cannot be fully controlled. The reagents and kits are produced by well-established manufacturers and sample volumes need to be high to ensure that the development of any given method on an immunochemical platform is profitable. Even though some immunoassays have been used for DBS as matrix[68,69], it has not been the first choice of analytical platform when developing a DBS method[70,71].

The use of DBS methods for the quantitative measurement of drug levels was not common prior to early 2000 due to limitations in instrument sensitivity when measuring concentrations from small volumes (≤30 μL)[72]. The development of more sensitive LC-MS/MS instruments has been one of the strongest facilitators for making DBS possible as matrix for TDM[7,72]. The growing interest in using LC-MS/MS methods in the clinical laboratory is likely due to their increased selectivity over immunochemical methods and the possibility to simultaneously analyze several compounds in the same analytical run[73]. All the bioanalytical methods for DBS in this thesis were developed using LC-MS/MS[73].

In adsorption LC, the analytes are separated from each other through interactions with a stationary phase and a mobile phase. Depending on the polarity of the analyte and the mobile phase, more or less retention on the stationary phase is achieved. After passing the column, the mobile phase with the analytes is led into the electro spray ionization (ESI) interface. High voltages, temperatures and drying gas assist in the ionization of the analytes. In the mass spectrometer, the molecular ions are separated, followed by fragmentation. Selected fragments, unique for the analyte of interest, are measured in a detector.

2.4 DBS IN THERAPEUTIC DRUG MONITORING (TDM) OF ANTIEPILEPTIC DRUGS (AED)

Clinical perspective

Over the last ten years, a multitude of bioanalytical DBS methods for different TDM analytes have been developed and validated [2]. Unfortunately, few of these methods seem to have been established for the processing of real patient samples as an alternative to plasma or whole blood. One reason may be that there is a lack of bridging studies, ideally with patients performing self-sampling, as these studies require knowledge, time and money. Another contributing factor is that plasma samples have been traditionally used and are well established and relatively easy to implement for hospitals/laboratories. Even though some clinical validations on DBS have been performed on commonly used AEDs[74–77], more clinical validations are needed to compare results obtained from plasma concentrations with capillary DBS samples gathered by patients at the clinic or at home[14,78].

For comparison of developed DBS methods for CBZ, LTG, LEV and VPA, see Table 1.

Table 1. Comparison of different DBS methods in humans, for CBZ, LTG, LEV and VPA

Author and year	Drug	Internal standard	Several drugs	Filter paper	Analytical method	Analytical range, µg/mL	Extraction technique	Punch size	Collection by patients	Type of blood	Conversion factors	Cross validation
Abu Ruz et al (2010) [79]	LTG	metformin	No	Whatman 903	HPLC-UV	0.5-20	Extraction w ethylacetate:1M NaOH (24:1), 3 min of vortexing, nitrogen dried and redissolved in mobilephase	6 mm	No	10 µL venous	No significant difference from plasma	No
Shah et al (2013) [42]	CBZ LTG LEV	hexobarbital	Yes	Whatman 903 Schleicher& Schuell	HPLC-UV	1-20 1-20 2-50	Extraction w MeOH:MeCN (3:1), 15 min sonication, nitrogen dried and redissolved, SPE, nitrogene dried and redissolved in mobilephase	6 mm	No	30 µL venous	Not studied	No
Rhoden et al (2014)[77]	VPA	cyklohexane carboxylic acid	No	Whatman 903	GC-MS	5-250	Extraction w MeOH:MeCN (3:1), 1 h sonication, extracts injected	6 mm	No	50 µL venous	1.883	Yes n=17
Kong et al (2014) [74]	CBZ VPA	5-p-methylphenyl-5-phenylhy-dantoin	Yes	Whatman 903	GC-MS	0.5-120 0.5-120	MeCN:NaOH 1M (24:1), 1 min vortex, 5 min sonication, nitrogen dried, derivatization, addition of heptane	6 mm	No	30 µL venous	1.95 (VPA) 1.01 (CBZ)	Yes n=182
Pohanka et al (2014) [75]	VPA	heptanoic acid	No	Whatman 903	LC-MS	1.4-173	Extraction w MeOH, 60 min of agitation.	8-9 mm	No	20 µL venous	DBS conc. 25% lower than plasma conc.	Yes n=32
Martins et al (2014) [80]	CBZ	propanolol	Yes	Whatman 903	HPLC-UV	0.63-?	Extraction MeCN:MeOH (1:3)during 30 min agitation. Evaporated and redissolved in mobile phase.	8 mm	No	20 µL	~1	Yes n=10
Shokry et al (2015) [76]	CBZ	CBZE-d10	Yes	Whatman 903	LC-MS/MS	1-40	Extraction in MeOH:water:formic acid (80:20:0.1, v/v/v),orbital shaker for a period of 25 min at 37 ◦C. 96-well plate	3.2 mm	No	20 µL venous or capillary?	DBS conc. ~23-30% higher than plasma	Yes n=19
Das et al (2017) [37]	CBZ	nevirapine	No	Whatman cellulose filter paper	HPLC-UV	2-20	Extraction in MeOH/MeCN/H2O (1.5 ml+1.5 mL+0.75 mL) for 15 min + 1 min vortexing	Whole spot was used	No	20 µL venous	DBS conc. ~20 % higher than serum	Yes n=80

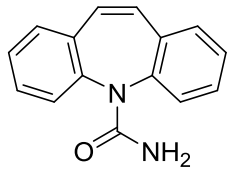
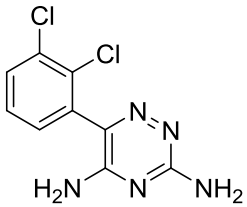
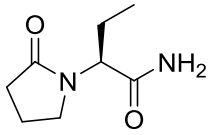
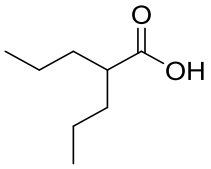
CBZ=carbamazepine DBS=dried blood spot LTG=lamotrigine LEV=levetiracetam VPA=valproic acid, MeOH = methanol, NaOH = sodium hydroxide MeCN = acetonitrile

2.4.1 Antiepileptic drugs

Around 50 million people in the world live with epilepsy, being the fourth most common neurological problem after migraine, stroke and Alzheimer´s disease[81]. The portion of the general population that have active epilepsy is somewhere between 4 and 14 per 1000 people, with the higher number referring to low- and middle-income countries[82]. In Sweden approximately 60 000 people have epilepsy and about 12 000 of these are children, 0−18 years old[83]. More than 70% of children and adults suffering from epilepsy can be successfully treated with AEDs which is the most common way to treat epilepsy. The treatment can either be life-long or prophylactic during several years.

The goal of AED treatment is to control seizures with minimal adverse effects, preferably through drug monotherapy. Some AEDs are also increasingly used for the treatment of bipolar disorders, borderline personality disorder[84,85] and neuropathic pain[86]. The chemical properties of the drugs covered in this thesis, CBZ, LTG, LEV and VPA are shown in Table 2.

Table 2. Chemical properties of four antiepileptic drugs

Analyte	Carbamazepine	Lamotrigine	Levetiracetam	Valproic acid
Molar mass (g/mol)	236.3	256.1	170.2	144.2
Monoisotopic mass (g/mol)	236.095	255.007	170.106	144.115
Molecular formula	$C_{15}H_{12}N_2O$	$C_9H_7Cl_2N_5$	$C_8H_{14}N_2O_2$	$C_8H_{16}O_2$
Structure				
pKa	13.9	pKa1 8.53 pKa2 9.21		4.8

Children with epilepsy can suffer from a wide range of syndromes, seizure types and underlying etiologies. TDM is essential for some AEDs and can be valuable in dose adjustments, particularly with drug combinations where drug-drug interactions are an issue. Some of the AEDs have complex intra- or inter-individual variations in pharmacokinetics, adverse effects and drug-drug interactions[87]. Children have higher plasma clearance of AEDs than adults and the clearance changes throughout childhood[26]. The complexity of epilepsy and the corresponding prophylactic treatment (especially in drug polytherapy) make TDM for AEDs an important tool for a better treatment outcome[88] even though it has not been proven that TDM is an effective tool for all AEDs[89].

In 2017, a meta-analysis study was carried out with the purpose of finding the drugs with best evidence to be recommended for monotherapy of various forms of epilepsy[90]. CBZ, LTG and

LEV are all suitable first-line treatments for partial onset seizures. For individuals with generalized tonic-clonic seizures VPA is a first-line treatment but avoided if possible in women of reproductive age[91] LTG and LEV can be used as alternatives. Essential information for each recommended drug studied in this thesis is presented in Table 3[26,87,92,93].

Table 3. Pharmacology data for four antiepileptic drugs used to control epilepsy in children

	CBZ	LTG	LEV	VPA
Year of approval in Sweden	1965	1994	2000	1981
Type of epilepsy most often treated with this drug	focal seizures, secondary generalized seizures	focal and generalized seizure in children w Lennox-Gastaut Syndrome, juvenile myoclonic epilepsy, infantile spasms, absence seizures, Rett syndrome	idiopathic partial onset or generalized epilepsy, juvenile myoclonic epilepsy	generalized epilepsies, focal seizures,
Common adverse events	drowsiness, nausea, dizziness, ataxia, hyponatremia	dizziness, sedation, headache, ataxia, double vision	somnolence, depression, behavioral abnormalities, headache, anorexia	dizziness, weight gain, vomiting, tremor
Serious adverse events	skin rash, Stevens-Johnson syndrome, hepatic toxicity	skin rash, Stevens-Johnson syndrome, toxic epidermal necrolysis, increased risk of suicide	behavioral changes	fatal hepatotoxicity, acute hemorrhagic pancreatitis, encephalopathy
Maintenance dose: (children)	10-20 (mg/kg/day)	1-10 (mg/kg/day)	10-20 (mg/kg/day)	20-40(mg/kg) (2-3 times/day)
Half-life (h)	10-13h (in children)	7-45h* (in children)	5-6h (in children)	8-13h (in children)
Therapeutic range	4-12 µg/mL (20-40µM)	2.5 -15 µg/mL (10-55 µM)	12-46 µg/mL (35-120µM)	50-100 µg/mL (350-700µM)
Bioavailability	75-85%	98%	>95%	>90%
Metbolism/ enzymes	CYP3A4, CYP2C8	UGT1A4, UGT1A1, UGT2B7	Only small amounts of hepatic metabolism	Extensive complex liver metabolism. CYP and UGT enzymes involved.
Main metabolite	CBZ 10-11 epoxide^	2-N-glucuronide conjugate	UCBL057	VPA-glucuronide
Protein binding in plasma	75%	56%	3%	74-93%
Urinary extraction	<1%	~10%	66%	1.8±2.4%
Blood/Plasma ratio[a]	0.95 to 1.05	1.1 to 1.3		0.51 to 0.70
Drug-drug interactions including CBZ, LTG, LEV and VPA	co-medication with VPA, risk of toxic levels due to increased CBZ epoxide.	co-medication with VPA increase the levels of LTG, co-medication with CBZ reduce the levels of LTG	CBZ and LTG enhance the metabolism and LEV concentrations can be reduced.	CBZ and LTG give rise to enhanced elimination and plasma concentrations of VPA are decreased.

a=ratios were experimentally derived from own experiments and are depending on the hematocrit level (see paper II) *=dependent on co-medication ^=active metabolite
References for the Table:[26,87,93,92]

There is a scarcity of bridging studies or cross-validations of DBS, including the AEDs in this thesis. DBS has been used for pharmacokinetic studies on CBZ (n=98) with blood from EDTA-tubes[94]. A study of adherence on five commonly prescribed AEDs was carried out with DBS samples collected at out-patient clinics and in some cases as self-sampling at home[95]. Incomplete sampling at home was mentioned as a problem. Other studies mostly developed methods for AEDs in proof of concept studies with few patient samples using venous blood collected in collection tubes. An overview of published DBS methods including, CBZ, LTG, LEV and VPA in humans is presented in Table 1.

2.4.2 Blood to plasma ratio

The distribution of drug concentration between the whole blood and plasma is referred to as the blood to plasma ratio. It is important to understand and study in relation to each drug before developing a DBS method[27,96,97]. DBS is in essence whole blood and the drug

concentration can be measured in whole blood instead of plasma as long as the unbound fraction of drug in plasma, the Hct and the partitioning are constant[27].

When the drug has a high affinity to plasma proteins, the partitioning into red blood cells is low and the blood to plasma ratio tends to be low ~0.55–0.60. VPA, is mainly distributed in plasma with a blood to plasma ratio of 0.64[96] which leads to lower concentrations in whole blood (including DBS) than in plasma[74,75,77].

The distribution between whole blood and plasma for CBZ and LTG is nearly equal with blood to plasma ratios close to 1. Therefore, direct comparisons have been proposed for these drugs[42,74,76,79,80]. For LEV there are no earlier studies where the relationship between plasma and DBS concentrations in humans have been investigated. The fraction of drug in the red blood cells and the fraction of drug in plasma can be investigated by an *in vitro* assay[98].

2.4.3 Conversion approaches

If a proportional bias between the drug concentration in plasma and the concentration in DBS is detected in the cross-validation, a conversion to an estimated plasma concentration can be calculated for the clinical interpretation[71]. Different approaches for this conversion are suggested in the literature. Theoretically, it should be possible to estimate a plasma concentration from the DBS concentration (C_{DBS}) by taking the individual Hct and the drug´s fraction in red blood cells into account. Such an equation has been proposed[98], where f_{BC} is the drug fraction in the red blood cells (Equation 1).

$$Equation\ 1. \quad \frac{C_{DBS}}{(1-Hct)}(1-f_{BC}) = Estimated\ plasma\ conc.$$

A similar approach was used to adjust DBS concentrations measured from VPA[74].

Another way of converting from DBS to estimated plasma is to use a mean ratio between plasma concentrations and DBS concentrations from a population and use this ratio as a factor (Equation 2). This approach requires data from clinical validations of many patients and awareness that extreme individual Hct can create bias. This conversion approach has previously been implemented for VPA[77].

$$Equation\ 2a. \quad \sum \left(\frac{C_p}{C_{DBS}}\right) \Big/ n = factor$$

$$Equation\ 2b. \quad C_{DBS} * factor = Estimated\ plasma\ conc.$$

2.5 DBS SELF-COLLECTION IN PATIENTS

Patient perspective

The use of DBS as a matrix opens the possibility of patient self-sampling at home. To our knowledge, DBS as alternative matrix for collection of samples at home for children with epilepsy has not been studied earlier.

2.5.1 DBS collection

Since collection of DBS has traditionally been performed by health care workers, DBS sampling guidelines have been developed [6,34]. If these guidelines are followed by self-sampling patients there is a good chance of quality in the collected sample.

It has been shown that instructions are crucial to be able to collect an acceptable DBS sample[99] and an effective way to instruct patients in practical techniques is the use of an instruction video[100–102]. Results have also shown that the participants wanted to have feed-back on performance[101].

2.5.2 DBS and self-sampling at home

Patient self-sampling has been performed successfully by different patient groups, for example in diabetes patients[19,103]. There are evaluated projects of self-sampling, although not with DBS, for international normalized ratio (INR) including self-sampling and monitoring[104–106] as an example of patients willingness to be responsible for both sampling and monitoring. There are also examples from screening for hepatitis or HIV with DBS[107,108].

DBS in TDM has been used in different studies where the patient self-sampled at home[20,109,110]. The majority of the adult patients preferred capillary sampling at home over venous sampling. DBS self-sampling at home in a child population was reported as feasible in one study[18]. In-depth studies on how guardians can collect DBS from their children at home are lacking.

3 AIMS

The general aims of this thesis were:

- Investigate the feasibility of DBS as a matrix for measuring concentrations of AEDs.
- Evaluate solutions for the use of DBS as a matrix in TDM of AEDs in children.
- To investigate the perspectives of guardians and nurses on capillary blood self-sampling of DBS.

The specific aims were:

Paper I: To study the matrix and to develop and validate an LC-MS/MS method for quantification of three common AEDs in DBS.

Paper II: To compare capillary DBS concentrations with plasma concentrations of CBZ, LTG and VPA in a child population and evaluate different approaches for conversion to estimated plasma concentrations.

Paper III: To develop and validate an LC-MS/MS method for four common AEDs, adapted to a clinical routine laboratory.

Paper IV: To compare capillary DBS concentrations with plasma concentrations of CBZ, LTG, LEV and VPA in a child population and evaluate if DBS concentrations collected by guardians could be used for TDM of AEDs in the future.

Paper V: Study how children with epilepsy and their guardians experience self-sampling of DBS and how their opinions and attitudes to self-sampling at home can assist to develop effective information and education for DBS self-sampling. Identify factors for successful DBS sampling.

4 METHODOLOGICAL CONSIDERATIONS

This chapter presents the background behind the methods used in the work and how these methods were chosen based on laboratory tests and other decisions in the course of this work. Sharing the methodological considerations behind the different studies is a way to understand the importance of methods and how much influence these method decisions may have on the results. Method limitations are discussed to ensure impartial and critical evaluation of the results and findings in this thesis.

Quantitative methods for bioanalysis have been combined with quantitative and qualitative methods related to patient opinions. Broad spectrums of research methods are covered to be able to achieve high quality data in the sampling-chain, from pre- to post-analysis. Even though a variety of methods are covered, the bioanalytical methods have been the main focus throughout this thesis. Although quantitative and qualitative methods focusing on the patient, provided valuable information, these methods are only partially described and not covered as thoroughly as they would have been in a thesis that had mainly focused on the patient's perspective.

Detailed information about materials and methods used in this work can be found in the material and method sections of the different papers, I-V.

4.1 OVERVIEW OF STUDIES

Method development and bioanalytical method validation have been the focus of this thesis. The designs for paper I and III are straightforward since they adhere to the guidelines for bioanalytical validation published by the European Medicines Agency (EMA)[111].

A level of complexity is added when patients and sample collections become part of the study design. In paper IV, data from two datasets were merged (including data from paper II) and the results were analyzed and presented together (Figure 5).

Overview of study IV.

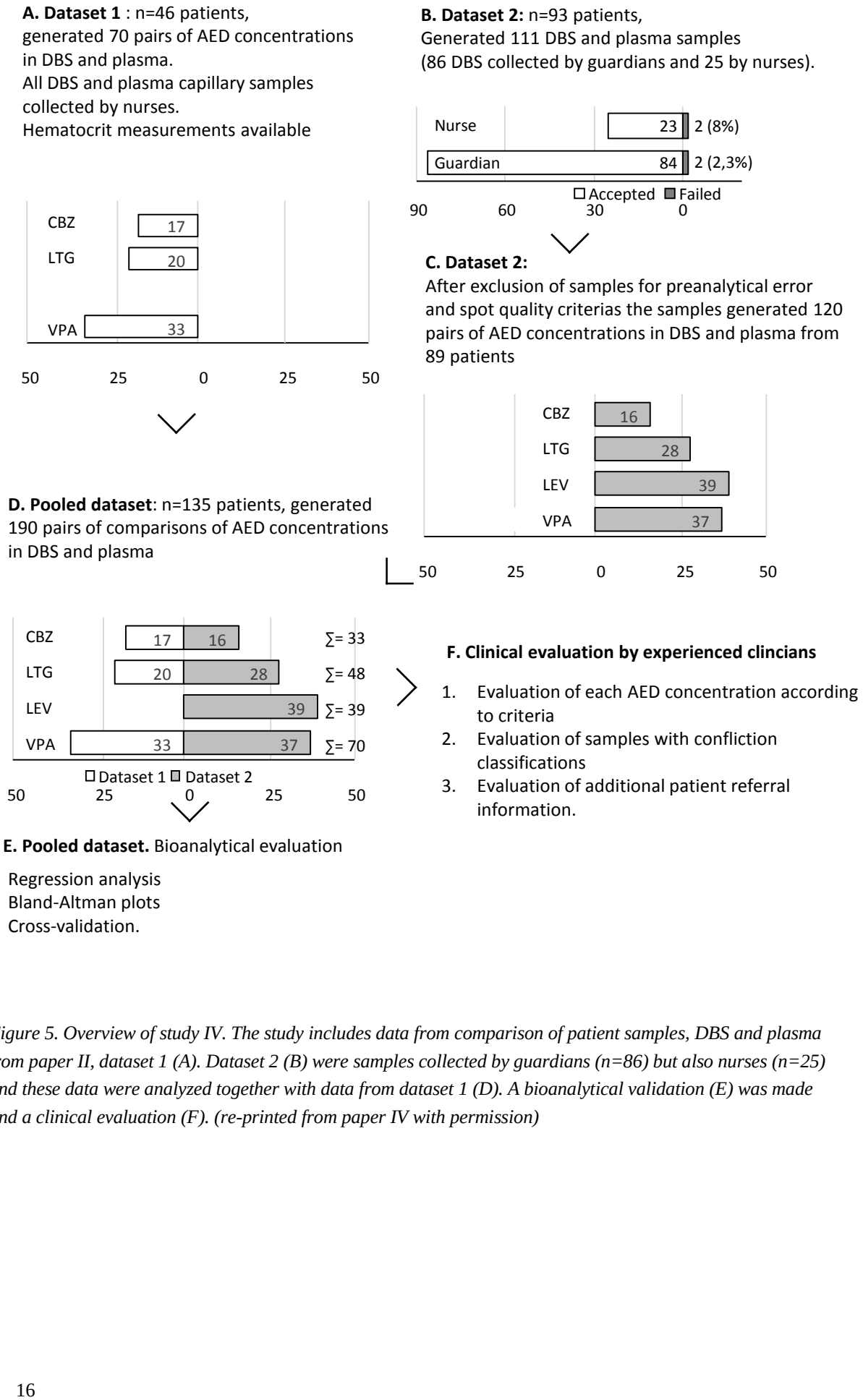


Figure 5. Overview of study IV. The study includes data from comparison of patient samples, DBS and plasma from paper II, dataset 1 (A). Dataset 2 (B) were samples collected by guardians (n=86) but also nurses (n=25) and these data were analyzed together with data from dataset 1 (D). A bioanalytical validation (E) was made and a clinical evaluation (F). (re-printed from paper IV with permission)

During the work with paper V, both quantitative (questionnaire) and qualitative (interviews) methods were used and the time period for this study was several years also including production of instruction material for sample collection (Figure 6).

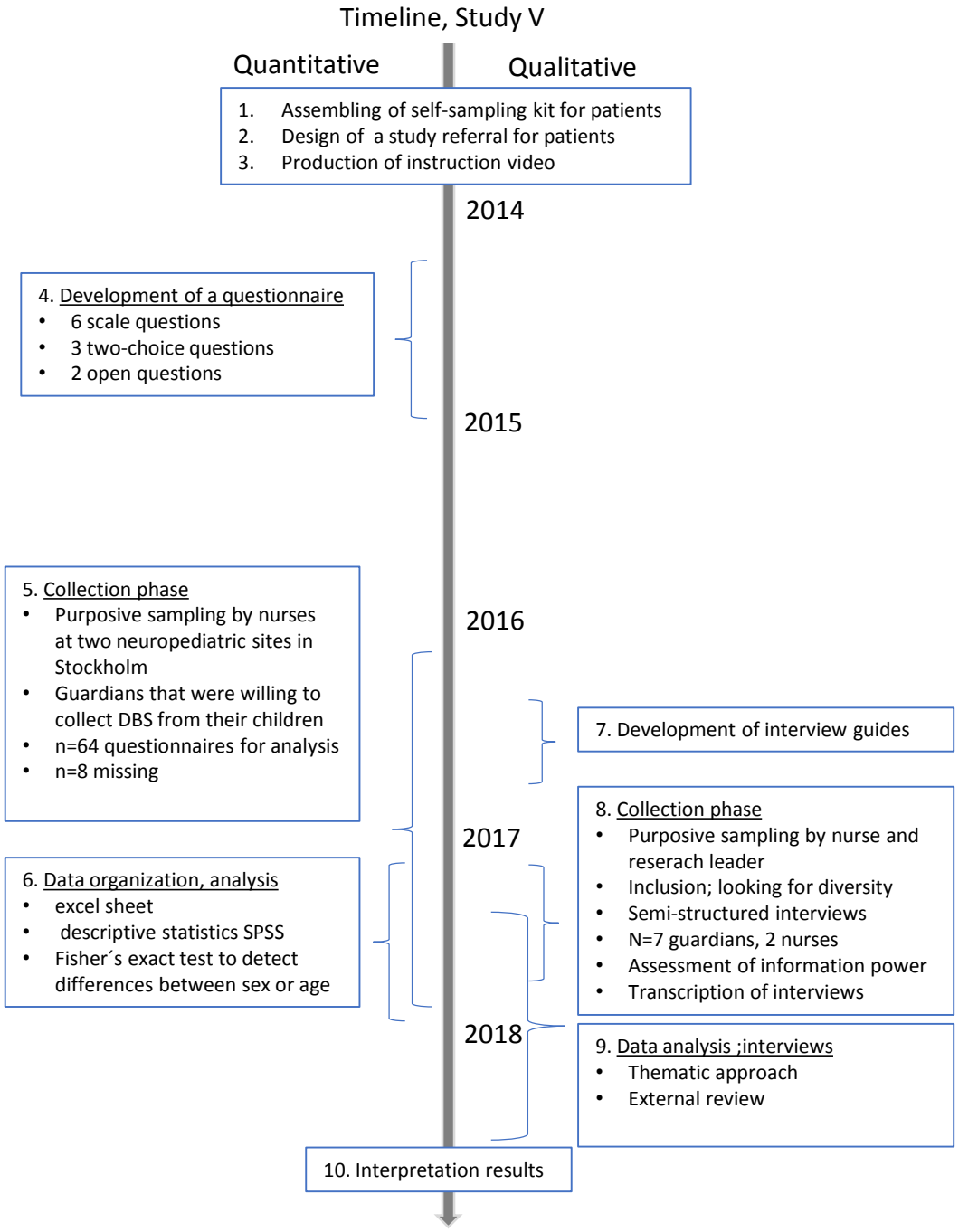


Figure 6. Timeline and overview of study V.

4.2 BIOANALYTICAL METHOD DEVELOPMENT

Laboratory perspective

Developing methods for measuring concentrations of different AEDs collected as DBS samples was new to the laboratory. The development can be described as an iterative process including the review of the scientific literature, discussions about the method, and decision-making regarding the type of experimental progress. The goal was to develop rapid, selective, sensitive and robust methods that could be readily implemented in a clinical routine laboratory.

4.2.1 Experiments during method development

In the methods described in paper I and III, a part of the blood spot with a defined diameter was punched out with a hole puncher. In paper I, a small handheld hole puncher with a diameter of 3 mm was used (corresponding to ~ 3.2 µL blood). A handheld hole puncher requires strength and good technique to punch out discs and they cannot be punched directly into a vial or a 96-well plate (Figure 7).

Figure 7. 1. Handheld hole puncher for 3mm filter paper discs from GE Healthcare. 2. DBS Wallac Puncher from Perkin Elmer with different punch sizes coupled to barcode reader and integrated with on-line laboratory information systems. Photo 2, courtesy of Perkin Elmer

In the development phase for paper III an automatic puncher, capable of punching directly into a 96-well plate was used. The puncher was coupled to a barcode reader (for sample traceability) and was capable of producing larger punches of 4.7 mm (corresponding to ~ 7.8 µL blood). A disc with a larger area has advantages since it contains more material and also to a greater extent equals out any inhomogeneity in the blood spot. By using a very small disc there is a risk of increasing the bias if the blood components are distributed unevenly on the paper[7,46].

In the development, paper I, different filter papers were examined. Whatman 903 A4 sheets, Whatman 903 Protein Saver Cards, Ahlström 226 and Whatman FTA DMPK-C cards, were used for measuring concentrations of spiked samples of CBZ, LTG and VPA. The goal was to find out if other filter papers were superior to Whatman 903 or if there were differences between the Whatman 903 A4 sheets and the Whatman 903 Protein Saver Cards, paper I. All the filter papers that were investigated consisted of untreated cotton linter papers (Whatman 903 produced in a slightly more acidic environment than DMPK-C[7]). Since chemically treated papers, such as DMPK-B, may interfere with the LC-MS/MS detection due to ion suppression, untreated filter papers are normally preferred for the quantification of small

biomolecules (<900 Dalton) using LC-MS/MS[14,38]. The non cellulose-based Agilent Bond Elut paper for dried matrix spotting was also tested with promising results, but the blood drops were difficult to deposit on the surface and the manufacturer reported that it was unsuitable for use by patients[112,113].

The Whatman 903 A4 sheets were used for creating calibrators and QC samples for most of the development and validations, whereas the protein saver cards were used for collection and analysis of patient samples. The comparison between the different filter papers showed no significant difference (CV ≤5.6% and accuracy within ±6%, paper I) and the conclusion was that Whatman 903 sheets and protein saver cards could be used as proposed for method development and validation.

Extraction of the analyte from the DBS is crucial and its efficiency can be evaluated by quantitative extraction recovery experiments usually carried out during method validation. For paper I and III different extraction solutions and techniques for extraction were investigated. Extraction with acetonitrile (MeCN) in combination with H_2O or methanol (MeOH) resulted in lower peak areas for CBZ, LTG and VPA as compared to extraction with pure MeOH. The extraction with MeOH and 20% H_2O (paper I) showed slightly higher peak areas for VPA. VPA is a weak acid that showed better ionization in negative mode than in positive and therefore was detected as negative molecular ion. Despite this, the sensitivity for VPA was not very good. It was concluded that the extraction was more effective for VPA when H_2O was present in the extraction solution and therefore the optimization was done mostly to improve conditions for VPA. Improved recovery with water in the extraction solution for some analytes has been noted by others[114,115]. Weakened interactions between hydroxyl groups on the filter paper and the analyte has been suggested as an explanation to increased recovery/efficiency with the addition of H_2O[116]. When the method was further developed for paper III, with the inclusion of LEV, the water content in the extraction solution was increased to 35%.

Ultra-sonication can increase the recovery at high Hct levels[55,117]. In paper I we reported recoveries of > 90 % and in paper III the process efficiency did not imply recovery problems. The liquid extraction with a step of gentle shaking was enough to extract efficiently. During method development extraction by ultra-sonication at normal and increased temperature was compared with normally extracted samples. The areas of the chromatographic peaks were not improved by sonication or heating.

4.2.2 The liquid chromatography method

LC-MS/MS methods can be simply understood as a separation step by chromatography and two separation steps and detection by mass spectrometry. The chromatographic method is more than just an inlet. By improving the chromatography it is possible to separate compounds in time and impurities in the matrix reducing the substantial matrix effects that would otherwise be a source of error for the mass spectrometry part of the system[118].

In paper I, we optimized the chromatography by choosing a column and a mobile phase that best suited the three analytes (Table 4). In paper III the chromatography method had to be adapted to be suitable for the routine laboratory, which limited the numbers of columns and mobile phases that could be used. In paper I the total run time for the method was 4 minutes and adapting the method for routine application required shorter runtimes (Figure 8). In the end, the gradient program described in paper III was suitable for all four analytes and retention times were robust despite a shorter run time of 3 min. In paper III the method was adapted to be able to focus and separate LEV, a relatively polar compound. With a vacuum centrifuging step, the extracts were dried and reconstituted in mobile phase A, containing 10% MeOH, which resulted in narrow peaks and stable retention times for LEV.

Table 4. Comparison of methods, paper I and III

Paper	I	III
Filter paper	Whatman 903	Whatman 903
Punch (d in mm)	3 mm	4.7 mm
Container	Glass vial	96-well plate
Extraction solution	200 µ of MeOH:H_2O (80:20 v/v)	200 µ of MeOH:H_2O (65:35 v/v)
Extraction	orbital shaker, 570 RPM, 5 min	orbital shaker, 450 RPM, 30 min
Reconstitution	No	mobile phase A:MeOH (90:10 v/v)
Instrument	Waters UPLC, Xevo TQ MS/MS	Waters UPLC, Quattro Premier XE MS/MS
Column	Acquity UPLC BEH C18 1.7µm, 2.1x100 mm	Acquity UPLC BEH, C18 1.7 µm, 2.1 x 50 mm,
Mobile phase A	2 mM ammonium acetate in H_2O	10 mM ammonium formate, 0.15 % formic acid in H_2O
Mobile phase B	100% MeOH	100% MeOH
Flow rate: mL/min	0.5	0.6
Injection volume µL:	2	4
Total run time:	4 min	3 min

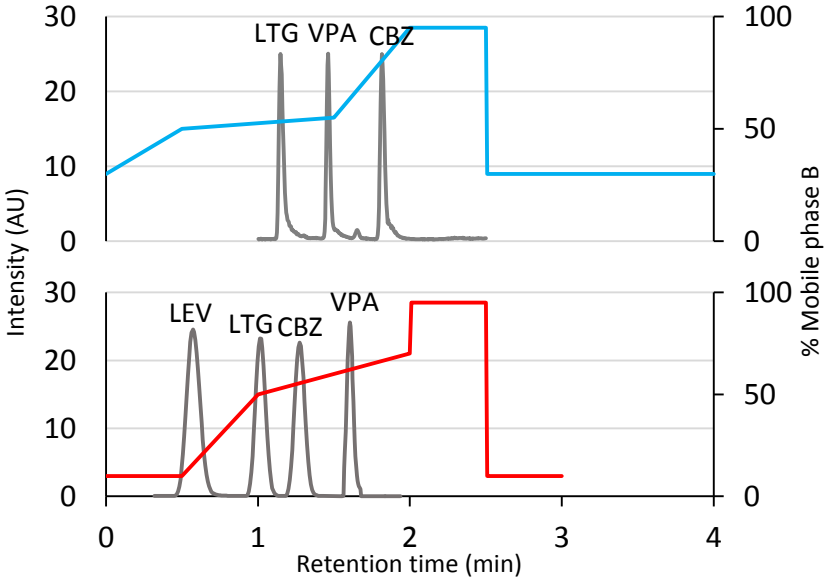


Figure 8. Visual graph of the gradient programs and retention times for the respective analytes in paper I and paper II.

4.2.3 The mass spectrometry method

A key factor when developing reliable mass spectrometry methods is the availability of isotopically labelled internal standards (IS). The IS is added to the sample early in the extraction process to compensate for any losses during the extraction and also for variations in the ionization process in the mass spectrometer. In the collision cell the IS is expected to fragment in the same way as the analyte. For DBS samples, the most straight-forward and common sample preparation method is to add the IS to the extraction solution. In this case, the IS does not compensate for losses during extraction since it is not incorporated into the wet blood[119].

Internal standards used in the methods (paper I and III) were deuterated for CBZ, LEV and VPA (CBZ-d_8 and d_{-10}, LEV-d_6, VPA-d_6) and carbon-13-labeled for LTG (LTG-$^{13}C_3$) , all of them stable internal standards that have similar retention time as their unlabeled counterparts[120].

During the tuning process, which aims to find the best precursor and fragment ions for each analyte, it was not possible to find stable fragment ions for VPA. Since the selectivity was not a problem, a possible solution for a TDM purposes was to let the precursor ion ($[M-H]^-$ *m/z* 143) pass through the collision cell with just a small amount of collision energy to guide it through with minimal fragmentation. Another possible option was to derivatize VPA, but this process would have been more labor-intensive in the sample preparation step. As a result of the low abundance of VPA ions, VPA had a longer dwell time than the other analytes.

Fine-tuning of the mass spectrometry parameters was done manually and compared to autotuning and the best options were used in the final methods. From the beginning more than one transition for each analyte was chosen. Before validation, however, the selection of transitions was narrowed down to the most suitable, usually the one with the most abundant fragment ion. Different injection volumes were tested and evaluated depending on calibration curves and their lower limit of quantification (LLOQ). The mass spectrometry settings of the two methods are summarized below (Table 5).

Table 5. Transitions and mass spectrometry settings, comparison paper I and III

Paper	Drug	$[M+H]^+$	$[M-H]^-$	Fragment	CV	CE	DT
I	CBZ	237.2		178.9	32	34	0.030
III	CBZ	237.3		194.1	30	10	0.050
I	CBZ-D_8	245.2		200.1	34	30	0.015
III	CBZ- D_{10}	247.4		204.1	30	10	0.050
I	LTG	256.0		144.9	46	38	0.030
III	LTG	256.2		211.0	45	27	0.050
I	LTG-$^{13}C_3$	259.1		159.9	50	28	0.015
III	LTG$^{13}C_3$	259.2		214.0	50	27	0.050
III	LEV	171.1		153.9	15	7	0.050
III	LEV- D_6	177.1		159.9	15	7	0.050
I	VPA		143.0	143.0	25	5	0.072
III	VPA		143.1	143.1	30	1	0.125
I	VPA-D6		149.1	149.1	25	5	0.015
III	VPA-D6		149.1	149.1	30	1	0.125

Settings	Paper I	Paper III
Capillary voltage (kV)	1.50	1.50
Source temperature (°C)	120	120
Desolvation temperature (°C)	600	350
Desolvation gas flow (N2) L/H	1000	800
Collision gas flow (Ar) mL/min	0.15	0.15

Ar=argon CE= collision energy CV= cone voltage DT= dwell time

4.2.4 Limitations and strengths during method development

In the method development for paper III, a limitation was the use of the same columns, mobile phases and wash solutions as used in the routine methods. One of the challenges was to solve different problems with the instruments, which require advanced skills. During this time period several different instruments were used and there were always small differences like tuning settings between instruments and day-to-day differences in instrument performance. An advantage is that in a routine clinical laboratory the staff always maintain the instruments in a structured and regular manner, thus minimizing the risks and reducing the possible bias that might occur if the maintenance was sub-optimal. Advanced technical and instrumental skills are crucial to be able to create a good LC-MS/MS method.

4.3 BIOANALYTICAL VALIDATION

In the effort to standardize bioanalytical method validation through the evaluation of various method parameters, EMA has published *`Guidelines on bioanalytical method validation´* [111]. The guidelines were followed in this thesis, with the addition of recommendations specific for DBS method validation[121].

Precision, accuracy, linearity, selectivity, carry-over, dilution integrity, matrix effect, stability and incurred sample reanalysis are all included in the full validation process according to EMA. Additional validation has been done for spot homogeneity, Hct effects, and stability in

extreme conditions, punch carry-over, reproducibility, effect of different spot volumes and capillary versus venous blood[49,121,122]. Validations were performed on two different instruments, with at least two biomedical scientists preparing the samples, during a prolonged period of time, thus making the validation process to mirror diverse conditions that are likely to occur in a routine laboratory over time.

4.3.1 Calibrators and quality controls (QC) samples

Fresh whole blood was used for creating calibrators and QC samples. Whole blood was gathered from volunteers and Hct was measured at the time of collection. In order to match the average Hct of 0.37 L/L found in the child population, blood was prepared to have an Hct of 0.40 L/L by adding or removing plasma after centrifugation[50]. Hct was measured once again before spiking and 0.40 ± 0.01 was accepted. Samples were always gently mixed on a rocker for more than 30 minutes before distributing the blood on the filter paper.

In paper III the stock solutions were prepared in dimethyl sulfoxide instead of MeOH for CBZ and LTG. The risk of precipitation when spiking whole blood was lower with dimethyl sulfoxide than with MeOH and LTG was easier to dissolve in dimethyl sulfoxide. Solvent volumes never exceeded 5% of the total volume in the working calibrators or controls. Blood from different individuals were never pooled as Hct measured from pooled EDTA blood differed significantly from the expected mean when batches of blood were tested. The blood was pipetted onto filter paper A4 sheets with calibrated pipettes.

4.3.2 Punch carry-over

Carry-over from the hole puncher needs to be investigated since it is usually not cleaned between samples. Carry-over from the puncher can be reduced by punching white filter paper between every sample. The carry-over was assessed by punching a high concentration spiked sample followed by three times punching of blank filter paper. From these tests it was concluded that punches of blank paper between the samples were not necessary (paper I and III).

4.3.3 Stability and extreme conditions

The idea of including extreme conditions in the validation for DBS is that the samples must be robust against hot, humid or cold mailboxes and transportation conditions. QC samples as well as patient samples were put in incubators in 50°C for up to 48 hours or at -20°C.

Analytes on filter paper can be hydrolyzed depending on humidity[123] but it has been shown that DBS are no longer affected after a drying time of 2.5 to 3 hours at ambient temperature[124]. Samples were always stored in zip-lock bags with desiccant packages to prevent high humidity. Robustness to humidity was not tested in the validation for these methods but it has been reported that the homogeneity of Whatman 903 was not affected by humidity when assessed by quantitative autoradiography studies[43] while others have shown effects of degradation in extreme humidity conditions[125].

4.3.4 Bias of different Hct and spot volumes on the concentration

Throughout the laboratory work, the impact on quantification by different Hct and spot volumes was extensively and repeatedly tested. In paper I different Hct levels between 0.20 to 0.60 L/L were tested and different volumes from 15 to 50 µL. In paper III, Hct levels and different spot volumes were investigated in the same test. The idea was to study possible combined effects on the concentration when combining, for example a small volume with a low Hct.

Patients being offered DBS self-sampling at home, should either have a normal Hct, or if in risk of being outside the normal range, a known Hct value so that he concentration bias can be corrected for.

4.3.5 Recovery, matrix effects and process efficiency

For LC-MS/MS methods it is important to evaluate if the sample matrix could introduce ion suppression or enhancement in the ion source. The analytes can compete with other co-eluting molecules in complex series of charge-transfer and ion-transfer reactions[126]. Competition between molecules in the ion source may thus affect the intensity of the desired ions. Since this process is matrix-dependent the evaluation of matrix effects is done with unpooled blank matrix (blood) from six different individuals[127], Figure 9. The six samples are spiked with low and high known concentrations of the analytes to create pre-extraction samples (A). Corresponding blank samples (same patients) are extracted according to the method and spiked to the same concentration used for the pre-extraction samples, thus producing post-extraction samples (B). The sets of pre- and post- extraction samples are then compared with neat solutions, of the same concentrations (C). The peak responses (area or intensity) are compared, and different ratios calculated to determine the extraction recovery (A/B ratio), matrix effects (B/C ratio) and process efficiency (A/C ratio), Figure 9.

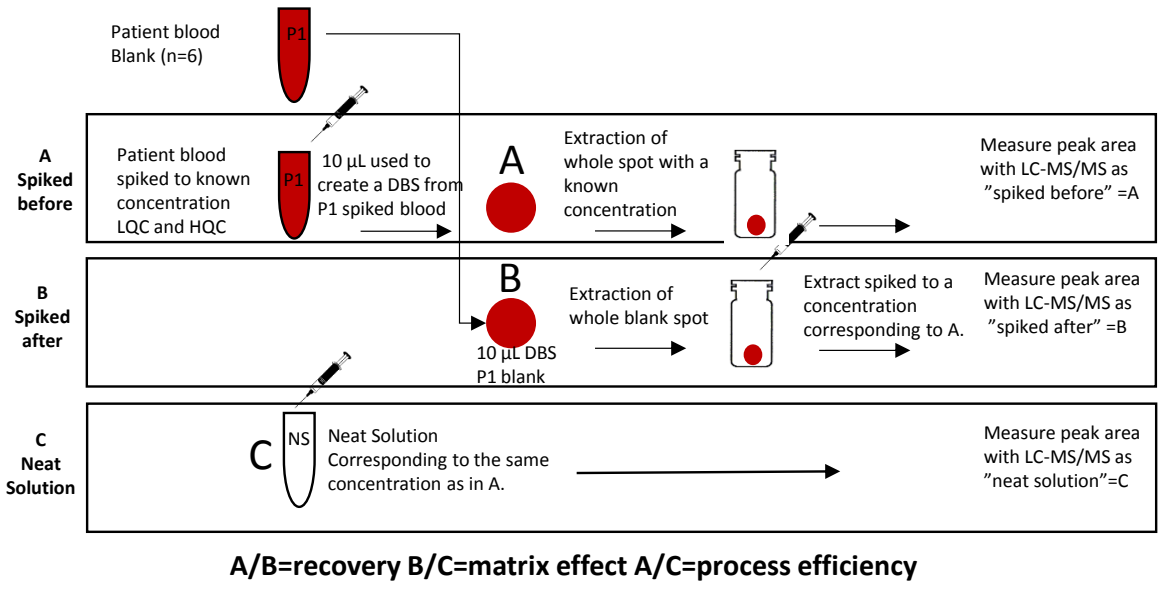


Figure 9. Experimental set-up for measuring recovery, matrix effect and process efficiency.

This evaluation has been carried out in different ways during the various validation processes. In paper I, the method was simple and whole spots containing 10 µL of pipetted blood from six different individuals were used. The extraction solution was scaled up to correspond to the same ratio between blood and solution as in the method.

In paper III, we decided to assess process efficiency exclusively (compare pre-spiked sample with neat solution). When only process efficiency is done (post-extraction samples not required), there might be a risk that ion enhancement can hide a low recovery.

Post-column infusion experiments are not often reported in validations but can be of importance to reduce the risk of interfering substances, e.g. phospholipids that may suppress the analyte signal[126]. In the post column infusion experiment no dynamic matrix effects were identified at the retention times of the analytes, paper III, Supplementary 1.

4.3.6 Capillary versus venous blood

Even though capillary blood is used in DBS for self-sampling at home, venous blood was used to prepare calibrators and QC samples in the validation process. Comparison of capillary versus venous blood was covered in the validation. Since capillary blood is a mixture of venous and arterial blood and the collection sites are different from each other, it is possible for differences between venous and capillary blood concentrations to arise[128]. It has been proposed, however, that any differences in measured drug concentration are likely to be noticed only shortly after drug administration[27]. For TDM, the sampling is carried out as trough concentrations, several hours after the drugs were administered.

Venous and capillary blood from three different individuals was each spiked at low and high concentration levels. Only small differences between spiked capillary and venous blood (precision and accuracy <10%) were seen for the four AEDs. This finding made it possible to use spiked venous blood for calibrators and QC samples instead of capillary blood.

Comparison of Hct between capillary blood and venous blood has not shown significant differences although greater variance between repeated sampling of capillary samples was found[129].

4.4 METHODS FOR COMPARISON BETWEEN PLASMA AND DBS CONCENTRATIONS

Laboratory and clinical perspective

The aim with the comparison of two methods would logically be to evaluate if they show the same results and can be used interchangeably. The comparison of DBS and plasma concentrations include a comparison between different matrices and different bioanalytical methods. It is likely that concentrations in one matrix differ from the other due to the blood to plasma ratio, and a factor can be applied to correct for this difference. There will be some variation in the factor and as the sample size increases, the confidence interval will be smaller. As described earlier, patient Hct can also have an influence on the result and to some

degree contribute to variation. In the end the most important thing to consider is if these variations might affect how the TDM answers for AEDs are used in practice

Several proposed limits for acceptance of method differences exist. The Australian Royal College of Pathologists, (RCPA) propose limits for most AEDs at ± 10% within the therapeutic range. Limits for cross-validation from EMA are wider. The difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats. EMA limits were considered to be suitable for these comparisons in a first step of clinical validation while the therapeutic ranges for CBZ, LTG, LEV and VPA are wide. It is recommended to use limits building on what is accepted in relation to how TDM is used for the specific drug. This approach was adhered to by performing a clinical evaluation after the clinical validation.

In this thesis, the approach has been to perform first a bioanalytical clinical validation and second a clinical evaluation. An accepted bioanalytical validation might still give rise to concerns; is the method good enough? To evaluate this, clinicians were involved to interpret if the variations between the concentration results should have generated the same recommendations. For the included AEDs, this was done by defining scenario categories for result classification

1. In a patient with poor effect, is there room for dose escalation?
2. In a patient with suspected adverse effects, is there room for dose reduction?
3. Is the concentration too high (potential of serious adverse effects)?

As long as the variations did not result in different outcomes in relation to these categories, they were judged to be acceptable. After the clinical evaluation, it was possible to decide whether the methods could be used and were valuable in TDM of AEDs for children with epilepsy.

4.4.1 Regression models

Methods for comparing measured concentrations from different matrices must be chosen. One of the most established methods for comparison between plasma concentrations and DBS is the Passing-Bablok regression[130,131], a non-parametric model insensitive to values from outliers. Constant and proportional bias can be estimated and analysis for linearity indicates significant difference from linearity.
The Passing-Bablok regression was used for comparison both in paper II and IV. In paper IV the interpretation was much less straightforward. In paper IV, the decision was to use simple linear regression and Pearson's correlation coefficient for presentation. Since the Pearson´s correlation only describes the linear relationship and not the agreement between data sets, Lin´s concordance correlation coefficient[132] test was also applied.

4.4.2 Difference plots

Bland-Altman plots are comprehensive and extensively used for visual presentation of the differences between measurements[133]. The plots were used to study differences, distribution, tendencies and outliers in the compared datasets. Since CBZ and VPA need conversion before concentrations can be compared, the converted DBS (CBZ, VPA) concentrations were presented in the comparisons. The Bland-Altman plots can be used either with absolute or relative values on the y-axis. Since the presentation with difference in concentrations on the y-axis is most often used, we decided to use that presentation and to add limits of ± 20% to be able to estimate the % bias in different concentration ranges in Figure 3, paper IV.

4.4.3 Conversion with Hct and K-value versus ratio approach

If the DBS concentrations differ proportionally to the plasma concentrations, it is possible to adjust the DBS concentrations to estimated plasma concentrations. An alternative to DBS estimated plasma concentrations from DBS would be to construct therapeutic ranges and decision limits for uncorrected DBS data instead of taking the detour over plasma concentrations. This approach calls for much more DBS data than is present at this stage as the power of clinical data from plasma over DBS is indisputable.

One method to create an estimated plasma concentration from a DBS concentration that was used in paper II was to include a K-value (the partitioning of drug into the red blood cells divided with the partitioning of drug into plasma) for the specific analyte and the individual Hct. This method for conversion was earlier used for VPA[74]. The methodological considerations are that individual Hcts and the K-value for each analyte must be available for this method of conversion and both of these parameters can be complicated to control, see section 2.4.2 and 2.4.3. The blood to plasma ratios for CBZ, LTG and VPA were investigated over the therapeutic concentration ranges and different Hct ranges in paper II (Supplementary). Blood to plasma ratios of the analytes were difficult to find in the literature and the idea was to investigate if there was a change in the ratio with different Hct and concentrations. Since the results showed that blood to plasma ratio varied, especially for VPA, it was difficult to use an equation for conversion with one average K-value (paper II).

Another way of conversion is to use the average ratio between the plasma concentrations and the DBS concentrations and multiply the DBS concentration with this conversion factor, see section 2.4.3. The advantage is that it is straightforward, but the disadvantage is that the conversion factor is easily affected by outliers if the population is small.

4.4.4 Limitations in the comparison

Results from the comparison between DBS concentrations and plasma concentrations were gathered and generated during an extended period of time. The risk of large differences increases with longer storage periods, different instruments in use, new reagents, kits and batches for the immunochemistry analysis (routine plasma samples) as well as the use of two different instruments and methods for the DBS concentrations.

Another limitation in the design was that the collected routine plasma capillary samples were stored for only two weeks. One possible explanation for some of the large differences in the comparison study (paper IV) might have been caused by erroneous answers from the routine plasma concentration. Since the DBS samples were analyzed at a time point when the routine samples were already discarded (more than two weeks after collection), there was no possibility to re-run the plasma samples if the results from the comparison differed more than expected.

It was difficult to collect enough patient samples for a clinical validation. At least 40 samples for comparison is recommended and this was especially difficult for CBZ[134]. During this time period it seems as other AEDs, such as LEV or oxcarbazepine, has been increasingly prescribed rather than CBZ[135].

4.5 MIXED METHOD

Patient perspective

For paper V, the choice of a mixed method design was decided in the analysis phase of the questionnaire data. The questionnaire gave information mainly on to what extent the guardians were satisfied with the instruction, the performance and if they were interested in self-sampling. The open questions were filled out by few guardians, contributing mainly with short positive sentences about the sampling procedure. In discussions with nurses at the neuropediatric clinics it was clear that the guardians had more experiences and opinions about this process than they wrote in the questionnaire. To be able to investigate the research question from different perspectives and to get a deeper understanding, one option was to make additional semi-structured interviews with informants that could contribute with rich and diverse information[136,137]. Such information has a greater potential to find problems and add value to patient education for DBS sampling, than the descriptive information from the questionnaire.

A mixed-method sequential explanatory design[138] was an approach that was suitable, because answers from the questionnaire could not be fully understood. One example was that ~70% had answered that it was easy to execute the DBS collection at the clinic but >80% thought it would be easy to collect at home (Supplementary 1, paper V). Why did the guardians think it would be even easier to collect the DBS in the home environment? Many guardians (~60%) thought it was somewhat painful for their child, but they still seem to be attracted to perform self-sampling at home if possible. Interviews as a collection method had the potential to answer these questions. Mixed-method sequential designs are most often designed with a main focus on the quantitative part of the study[139]. We decided to give equal importance of the quantitative and the qualitative part.

4.5.1 Study subjects and data collection, paper V

Questionnaire

Inclusion criteria for the questionnaire were the same as for participating in the DBS comparison project (paper IV). Nurses were requested to ask patients (children with epilepsy and their guardians) when they were scheduled to collect a blood sample for measuring AED concentration. On this occasion the nurses asked if the family was interested in participating in the study. Families that showed an interest in future self-sampling at home and understood Swedish, received written and verbal information. All guardians and participants ≥12years signed a written consent. Children under 12 were informed by nurses and guardians and if a child refused or if there was a sign of unwillingness to cooperate, the self-collection was not carried out. The questionnaire was handed out together with the blood collection kit and filled out by guardians or older children who had sampled themselves, immediately after the DBS blood collection.

Interviews

Two interview guides, were developed in the study, one for guardians and one for nurses. In a first phase research questions were defined and areas of interest related to the question, were created in the interview guide. The guide with questions were tested on nurses and one guardian in the development phase. Five main areas, related to experiences and feelings that informants had during the sample situation, were covered in the interview guide. 1) the education 2) feelings before the blood sampling collection 3) the sampling and the blood collection 4) the communication and relation between child and guardian. A fifth area, not related to the actual sampling procedure, considered the participant's thoughts about the future and performing home sampling. The interview guide for the nurses covered the same main areas, except number 2, but also how the nurses evaluated their own role in this situation. A total of nine interviews were performed and analyzed (seven guardians and two nurses, purposively chosen by the author and a research nurse in the team) .

Since all the guardians included for interviews had performed the sampling themselves, all had experiences and feelings related to the questions. The included nurses had experiences from being educators in the self-sampling situation and they had valuable information and an overview perspective since they had seen many guardians collect DBS samples.

The informants were encouraged to give detailed and rich descriptions. The interviewer used different kinds of tools to make the informants share their experiences and feelings, for example warm-up questions, follow-up questions where the guardians were asked to exemplify, clarifying questions and closing questions with a summary to seek agreement in what was said. The interviews ended when a sense was achieved that the whole picture was addressed[140].

In qualitative research the term "saturation" is often mentioned in relation to sample size and is referring to a whole methodology[141]. In the work with the qualitative method used in paper

V, interviews were performed and analyzed in a process. When no more categories emerged from the data, two additional interviews were performed and after this saturation was assumed. Another model to guide the judgement of sample size in qualitative research has been proposed[142]. The model is called "Information Power" and takes into consideration several items and dimensions that can increase the information power, which means that smaller sample is needed. For example, a narrow aim of the study, if the participants hold characteristics highly specific for the study, an established theoretical background, high quality of the interview dialogue (interviewer experience) and analysis strategy (case versus cross-case) are items that can increase the information power. This model was discussed in relation to sample size during the work with paper V.

The transcribed interviews were handled as confidential material and should not be able to reveal the identity of an informant. Participants' lists, audio recordings, questionnaires and transcribed interviews are kept in a locker so that no one without permission can get access to the data. Participants were informed of their rights to withdraw without an explanation at any time during the study period and that their decision of participation or not, would not affect their treatment.

4.5.2 Methods for analysis of quantitative and qualitative data

Questionnaire

The primary focus of the questionnaire was to give feed-back on the given information, (practical from nurse and the video instruction), of the blood collection process and to estimate the preference of self-sampling at home. The questionnaire was developed by the research group and iterated between nurses, pediatricians and researchers before finalized.

The aim was to use descriptive analysis mainly to get feed-back from the guardians and use the information to improve the instruction video and the education situation. From the beginning there was no intention to analyze correlation between for example age of the child, and successful sampling or preference for self-sampling at home. In the analysis phase some of the questions were investigated in relation to the age of the child with Fisher´s exact test. The idea behind this was that it was interesting to understand if it was more complicated to collect samples from smaller children than older and therefore two age groups were formed, ≤4 or >4 years old.

Interviews

Thematic analysis was chosen as a method for organizing and interpreting data from the interviews. This method to analyze the data has a tradition in health services research and has been used to find diverse perspectives of studied phenomena[143,144]. In paper V the analysis process has been inspired by Malterud and systemic text condensation[144]. The method is structured with guiding steps and said to be suitable for nurse students or others with limited earlier experience from analyzing qualitative data[144]. Thematic analysis is also a method that give accessibility and flexibility and a suitable entry to qualitative research for beginners[143].

The analysis phase is an ongoing and iterative process where the researchers are immersed in the data. The process also means repeatedly moving between different stages in the data. This requires structure and organization of the different stages in the process. In the analysis for paper V the data and the different steps were iterated, organized, structured and documented through the whole process.

4.5.3 Limitations (mixed-method study)

The questionnaire, quantitative method

The number of families that were asked to participate in the study (questionnaire) was not registered. It is therefore unknown how many of the families who were asked for participation and how many that declined. There is a risk of bias, for example that the data about the guardians' education level or socio-economic background was not collected can create selection bias and the studied group may be different from the rest of the guardians interested in performing self-sampling at home.

Another limitation was that it was difficult to find information on the total numbers of children/families listed at the two neuropediatric clinics that were involved in the study. To get an estimation of how many patients were included, we compared it with the number of prescriptions of the four AEDs (0-19 years old) that were registered as collected at the pharmacy in Stockholm Health Care Region (SR) in 2016 by the national drug prescription register [135]. From this register it was found that 72 patients correspond roughly to 3.5% of the total patients (0-19 years old) treated with these AEDs (SR). Since >15% children were on drug polytherapy it means that the study population represents more than 3.5% of the possible patients in SR in this age group. The four AEDs covered 92% of all the AED prescriptions in SR.

The children and their guardians were from two major university hospitals in the SR region and therefore the pediatricians might have more access to research than other Swedish regions. On the other hand, there are national recommendations by the Swedish Medical Products Agency on how to treat children with epilepsy and all should be treated by and in contact with specialized children neurologists and AEDs are recommended according to guidelines. The study design was limited to the Stockholm Health Care Region and to include other regions in Sweden would have strengthened the transferability.

The initial aim was to analyze the questionnaire with descriptive statistics, but it would have been interesting to also find out if there was correlation between for example problematic sampling related to concomitant diagnose and/or age. This aspect was not thought upon from the beginning and it was therefore difficult to analyze the questionnaire in relation to correlation hypothesis. Demographic data like age, sex and education level of the guardian and concomitant diagnose in the child was not collected and this information could have been valuable to be able to find interesting correlations in the material.

The interviews, qualitative method

Qualitative methods should be discussed in terms of data quality and validity, to ensure trustworthiness of the results[145]. During the process where meaning-units, codes, sub-categories and categories were formed, two researchers were involved. To increase validity, another independent research nurse analyzed the material. Furthermore, the members of the research group read the transcripts and confirmed that the results were rooted in the original transcripts. The citations used were another way of adding validity in analysis of the categories, paper V.

In qualitative research methods, credibility is important to create trustworthiness. The concept of credibility refers to the ability and effort of the researcher reflexivity, i.e. how much the researcher explains about her background and experiences and how this may affect the research, the ability to question the findings and interpretations and thinking about context and bias[136]. The principal researcher/author of this thesis, who also performed the interviews had a previous understanding from earlier experiences and contacts mainly with the nurses involved in the study and the sampling procedure but also from analyzing the questionnaires. Although it is difficult to bracket the preconceptions that resulted from these contacts, the researcher was conscious about her earlier experiences and that they may have influenced her opinion about the sampling situation. The author had no earlier experience from qualitative research but worked with an experienced qualitative researcher through all parts of the work.

The first interview had a face-to-face format with one guardian and was used as a pilot interview for the interview guide, and since the data qualified for answering the research question it was used in the analysis. The informants thereafter had the possibility to choose what best suited them and all the guardians chose telephone interviews for different reasons. The interviews with the nurses were face-to-face. There are advantages and disadvantages with these interviewing methods[140,146]. In a face-to -face interview it is easier to catch up on interesting things behind the words because it is possible to see and analyze facial expressions. On the other hand, it is important that the informants feel comfortable and free with a high degree of influence over the interview situation[147]. The telephone interview was a way of making the data collection easier to deliver for the informants.

Having had five mothers and only one father participating in the study (paper V) is seen as a limitation. The aim was to have a situation that was resembling the ratio between mothers and fathers who come to the clinics with their children. At least 40% of fathers as guardians would have been satisfactory. At the time of the interviews however, it was difficult to find available fathers.

How transferable are the findings in paper V[136]? The results can be of value in other areas in health care when blood samples from children could be collected in a home environment. The information from the study can be used to prepare parents, children and nurses and to produce effective instruction material for DBS sampling in a home environment.

5 ETHICAL CONSIDERATIONS

The principles of the Declaration of Helsinki[148], applicable to the research that has been conducted throughout this thesis, is briefly commented upon in this section.

Risks, burdens and benefits

In the study (II, IV and V) where children and guardians were asked to participate in DBS collection, burdens and risks, although small, have to be considered. For a couple of children, an extra fingerprick was necessary to complete the collection for both routine and -DBS collection. However, the design of the blood collection was done to avoid this by using the same fingerprick for both samplings. The children had to be still for blood collection during longer time than for routine capillary sampling, which can be demanding. For guardians, there was a risk of burden because they may feel that they harm their children by performing the fingerprick. The use of spring-loaded lancets makes it possible for unexperienced persons to fingerprick without risks. The risks and burdens for the individuals are considered to be small and have to be related to the benefits that may come with the research for this particular group, the possibility of performing self-sampling at home instead of collecting the sample at the clinic at the hospital. All individuals participated voluntarily and had the right to withdraw from the study at every moment. Information about the risks and procedures for blood collection was thoroughly described, both in written and verbal information.

Vulnerable groups and individuals

In the declaration of Helsinki, it says that research with a vulnerable group is only justified if the research is responsive to the health needs or priorities of this group and the research cannot be carried out in a non-vulnerable group. This study was made on a vulnerable group, children with epilepsy. One might ask why this research had to be carried out in this specific group? To use an adult population for the study had been possible if the research question had been limited to investigation of the matrix and its comparison to plasma. The drive behind this research has been from the perspective of these children and their families together with interest from the pediatricians. To find obstacles and to prepare this specific group for self-sampling at home could not have been done in an adult group.

Informed consent

All guardians and children ≥12 years old signed a written consent that was included in the ethical application. Effort was taken to have informed consent[149,150] from all children and guardians, meaning that the nurses involved in the study tried to inform the children in a way that they could understand. If a situation evolved with signs of misunderstanding or unwillingness from the child, the nurse recommended the guardian to not perform the fingerprick.

6 RESULTS AND DISCUSSION

In this section some of the most important results from the studies, paper I-V, are presented and discussed in relation to what can be confirmed or opposed from the literature. Reasoning of findings coupled to the initial aims is presented as to conclude and highlight what was new in this research and to what extent the findings will contribute to practice or future research.

Laboratory perspective

6.1 METHOD DEVELOPMENT AND VALIDATION

Throughout the work with this thesis, standard validation parameters as well as additional parameters specific for DBS were repeatedly investigated for CBZ, LTG, LEV and VPA on Whatman 903 filter paper.

Accuracy and precision were within ± 15 and ≤ 15% (LLOQ ± 20 and ≤ 20%)[111] for both methods developed in paper I and paper III, with slightly better results for precision in paper III than paper I. The therapeutic ranges for the AEDs were well within the quantification range of the analytical platform, Table 6. The methods were adapted to the conditions of the routine TDM laboratory, in relation to choice of columns, mobile phases and short runtimes, and the methods showed robustness and good accuracy and precision. Several full validations of the methods were undertaken during this time and results were repeatable.

Table 6. Method parameters of plasma analysis and DBS analysis, paper I and III
CBZ=carbamazepine, LTG=lamotrigine, LEV=levetiracetam, VPA=valproic acid

Drug	Matrix	Platform	Quantification range µg/mL	Therapeutic range µg/mL	Precision (%)		Calibration
					Within	Between	
CBZ	Plasma	CEDIA	1.18-19.8	4-12	1.2-5.2	4.1-5.2	
CBZ	DBS	LC-MS/MS	PI: 0.75-40 PIII: 0.59-18.9		1.6-5.1 4.8-6.0	3.1-15.3 4.8-7.5	Quadratic Linear
LTG	Plasma	QMS	1.28-12.8	2.5-15	3.1-9.2	4.5-13.9	
LTG	DBS	LC-MS/MS	PI: 0.75-40 PIII: 0.64-20.5		2.4 -11.7 4.4 -10.5	4.8-10.6 4.8-7.2	Linear Linear
LEV	Plasma	HEIA	2.04-100	12-46	4.2 -15.5	6.2-13.7	
LEV	DBS	LC-MS/MS	PIII: 0.83-67.7		5.5-7.8	4.9-8.5	Quadratic
VPA^	Plasma	LC-MS	14.4-225	50-100	1.5-2.7	1.5-4.8	
VPA*	Plasma	CEDIA	14.4-150		0.7-1.6	1.1-1.9	
VPA	DBS	LC-MS/MS	PI: 5-300 PIII: 2.90-145		2.7-7.5 5.4-9.7	4.7-11.8 4.5-8.8	Linear Linear

^=the plasma method used for comparison in paper II *=the plasma method used for comparison in paper IV, PI=paper I , PIII=paper III

We thoroughly investigated the effects of different Hct and drop volumes to enable the study of the combined effects. Results showed a trend of negative bias with small drops and low Hct and positive bias with large drops and high Hct (see Figure 10).

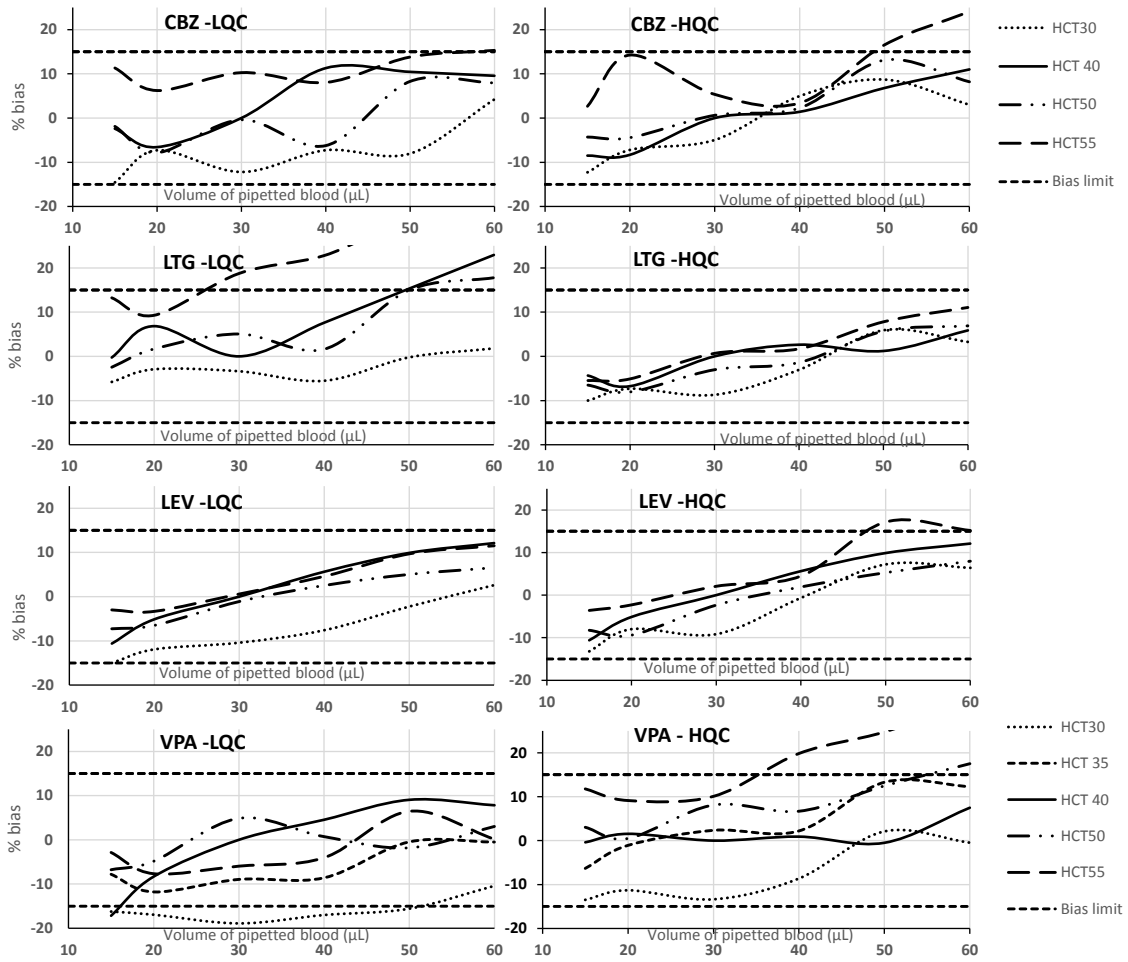


Figure 10. Combined bias (%) from Hct and volume on measured concentrations (modified from paper III, Supplementary information with permission).

This bias on the concentration was expected when using Whatman 903 filter paper in combination with punching a fixed diameter, see section 2.2.4 and 4.2.1.

In paper I, Hct levels and different drop volumes were examined separately. The results showed bias within ±10% for different volumes (15–50 µL) and within ±15% for Hct levels between 0.35–0.60 L/L. There was no additional effect noticed when Hct levels and volumes were combined, as performed in paper III. A bias greater than -15% was seen for LQC VPA at low Hct (0.30 L/L), Figure 10. On the other hand, this low concentration (LQC was 60 µM or 8.7 µg/mL) is below the lower therapeutic range for VPA and is therefore unlikely in patient samples. The approach of combination of different volumes and Hct in the same validation assay has not been applied in other published articles. Often the parameters are investigated one by one and additional or nullifying effects cannot be studied. Although, there are articles showing combined effects of Hct, concentration and recovery[151,152].

The method was validated for patient samples with drop volumes ranging between 15–50 µL in a Hct range of 0.35–0.50 L/L. The same results, with minor influence of different volumes but more for extreme Hct, has been identified by others[42,76,77]. No patient was excluded due to extreme Hct, paper II. In paper IV Hct values were not collected since the assumption was that no patients in the study would have extreme Hcts.

One might think that large differences between results in DBS (converted for CBZ and VPA) and plasma can be explained by very low or high Hct values in some patients and that the difference could be corrected with adjusted Hct. However, a correction for Hct in the patient samples only changed the results of the DBS concentrations with an average of 2.1% (paper II, Supplementary 7). Hct correction was not contributing to better agreement between methods and individual Hct values could not be used as an explanation for large differences in some of the sample pairs. On the other hand the individual Hcts in this population did not vary much and were all in the range of 0.32−0.42 L/L. A recent clinical validation of DBS for sirolimus and everolimus found that large differences in some of the samples could not be explained by the patient Hct[153]. Since the bias of extreme Hct can be large, it must never be neglected that extreme Hcts will affect the measured concentration on Whatman 903 filter paper and these types of bias must be avoided.

The correlation between differences in volumes of blood drops was also studied. Small or large drops of blood (volumes) in the patient samples did not explain any large differences between DBS and plasma results. When the concentration was measured in two different blood spots from a patient sample the mean CV% between spots was 3.2 (CBZ), 4.0 (LTG), 3.1 (LEV) and 3.9 (VPA), Supplementary 1, paper IV. Despite the different blood volumes deposited on the filter paper, the CV% was small.

When comparing peripheral to central punches, the peripheral punches had higher concentrations than the central with a mean bias of 8.2−11.0 % higher concentrations in the peripheral spots for the different AEDs, paper I, Table 7. This inhomogeneity on Whatman 903 paper has also been reported for other DBS methods and analytes with essentially the same results[46,47,51,154]. The result of inhomogeneity in the spots is considerable and emphasizes that it is important to always sample the same location within the spot to prevent additional bias. As long as a central punch from the filter paper is used, it is possible to limit the bias from the chromatographic effect. Whole spot analysis would be more representative of the blood sample[155] but is not possible with collection of blood from fingerpricks on Whatman 903 filter papers when the volume of the drops is unknown.

Results showed that CBZ, LTG, LEV and VPA were stable in DBS at room temperature for up to one year, Table 5, paper III. No other studies have shown results for such long storage periods of DBS at room temperature for these drugs. Precision and accuracy were acceptable following storage at temperatures as high as 50°C and as low as -20°C. The results demonstrated that DBS is a robust matrix for these analytes and can resist harsh conditions since no trends were noted for any of the analytes and the accuracy was within ±15%, with only LTG HQC having +15.6% deviation from the nominal concentration after storage at 50°C (in Table 5, paper III). We could thus show that patient samples can be transported in envelopes and stored in mailboxes for several days with a low risk of affecting sample quality.

The stability of LEV in fresh whole blood is affected by enzymatic degradation during storage in whole blood before separation of plasma[156]. Thus, it would be interesting to investigate if DBS may be a better choice than plasma when analyzing LEV

Recovery and matrix effects have been studied in paper I and III, both in qualitative (post-column infusion) and quantitative experiments. Results showed no dynamic matrix effects at the expected retention times of the analytes.

In the validation for paper I, recoveries were between 93.6 −104.9% and matrix effects were within 98.7–116.3%, CV% 1.5–4.1 for all analytes (Table 4, paper II). Impaired recoveries have been noted with high Hct samples by others[55,152] but in the investigation for paper I, we found that the high recoveries for CBZ, LTG and VPA were maintained also at high Hct, (0.60 L/L). In Table 4, paper III, process efficiency was between 90.1− 96.4% (CV% 2.6 − 4.4%), indicating high recoveries and low matrix effects.

In conclusion, rapid and robust LC-MS/MS methods for measuring the concentration of CBZ, LTG, LEV and VPA in DBS have been developed with precision and accuracy that were in the same ranges as the routine methods. The methods were accepted according to the criteria for method validation from EMA, when performed in a normal Hct range.

6.2 BRIDGING STUDIES AND STATISTICAL TESTS FOR AGREEMENT

Clinical and Laboratory perspective

Bridging studies for DBS and plasma were performed to evaluate if the methods could be used interchangeably[157].

In paper II and IV, Passing-Bablok tests showed a clear proportional bias for VPA, which indicated a need for conversion into estimated plasma concentrations. For CBZ, the Passing-Bablok tests did not indicate a need for conversion since the confidence interval of the slope included 1. Measured concentrations of CBZ in DBS, however, were constantly higher than those measured in plasma (paper II and IV). The conclusion was that DBS CBZ concentrations would be in better agreement with plasma concentrations after conversion.

6.2.1 Conversion factors

After investigating different conversion approaches (paper II), the conclusion was to use the ratio approach, where a factor was calculated from the average DBS and plasma concentration ratios. The factor for CBZ was 0.84 in paper II and 0.85 (standard deviation 0.10 for both) for the pooled dataset in paper IV. In other DBS plasma cross-validations for CBZ, higher DBS than plasma concentrations were reported in line with our results[37,76] . Other groups found no difference between the matrices for CBZ[74,80], Table 1. None of these cross-validations were comparisons between capillary fingerprick blood and plasma but were based on venous DBS. In paper II and IV all data presented is from capillary blood samples from a fingerprick directly deposited on the filter paper, which is the true matrix to be studied in a clinical validation for DBS.

The choice of a correct factor for conversion was more challenging for VPA since the factor varied from 1.58 (standard deviation, 0.16) in paper II to 1.64 (standard deviation 0.20) in the pooled dataset, in paper IV. Different values of factors from small cross-validations have also been reported[74,75,77], Table 1. In the pooled dataset (in paper IV) the ratio was calculated based on more samples, leading to a higher factor which is considered more accurate and thus the factor suggested for the conversion of VPA DBS concentrations. The variation in conversion factor does not seem to affect the clinical evaluation of the samples for VPA.

6.2.2 Tests of agreement

Lin´s concordance coefficients[158] were between 0.901− 0.954 for the four analytes, after conversion for CBZ and VPA (paper IV, table 1), which can be interpreted as moderate to substantial correlation[132]. Additionally, the Bland-Altman plots showed that 89% of CBZ and 93 % of VPA comparisons were within ± 20 % (paper IV, Figure 3). There were, however, deviations greater than ± 20 % in several comparisons for LTG and LEV, although the acceptance limits for cross-validation from EMA were met.

For LTG, the differences in the therapeutic range were demonstrating that LTG DBS concentrations were higher than the measured plasma concentrations, except in the low range where they were lower, paper II, Fig 2 and paper III, Fig 3. One solution to achieve better agreement in the mid and higher range would be to use a factor also for LTG. A conversion with a factor would have a negative effect in the lower range (<3µg/mL), since the bias for LTG was concentration dependent.

6.2.3 Effects of different Hct in the child population

The mean measured Hct in capillary blood in this child population was 0.37 L/L (standard deviation 0.025) and the bias related to Hct was negligible, (paper II). When calculating an estimated plasma concentration in paper II, we concluded that the individual Hct could be replaced by an average Hct of 0.37 L/L since the concentration (µg/mL) differences after Hct adjustment were small, mean difference in % for CBZ was 0.14, LTG 0.43 and VPA 3.14, see Supplementary 7, paper II. Extreme Hct values can still give rise to bias outside the accepted limits, thus children expected to have deviating Hct should not be candidates for home sampling.

6.2.4 Clinical evaluation

The studied population was children between 2−18 years old with a mean age of 9. Approximately 15% were on polytherapy, with VPA+LTG as the most frequent combination. The population covered different seizure types and ~30% had different concomitant diagnoses

In paper IV, three clinicians did an evaluation of 190 sample pairs in three steps by independently sorting the different concentrations into different categories and finally judging if the recommendation of drug treatment, based on a DBS or a plasma concentration, would have differed for any of the patients included in this study. As described previously (section

2.4.1), the included AEDs have broad therapeutic ranges and the drug treatment must be related to the individual patient considering possible co-medications that may cause drug-drug interaction effects.

Results of the clinical evaluation showed that difficulties with different recommendations arose mainly in the concentration range where there is a risk of adverse effects, Figure 11. From a total of 190 comparisons, two patients on LTG (4.2%) and two patients on VPA (2.9%) could be at risk of obtaining different clinical interpretations, depending on which method (DBS or plasma) that was used, see Table 2 in paper IV. From a clinical point of view these differences were not dramatic. As a precaution for patients that wish to choose self-sampling with DBS, a follow up sample in plasma is recommended if the DBS result deviates from expected concentration or if there is a sign of adverse effects, especially at critical concentration ranges.

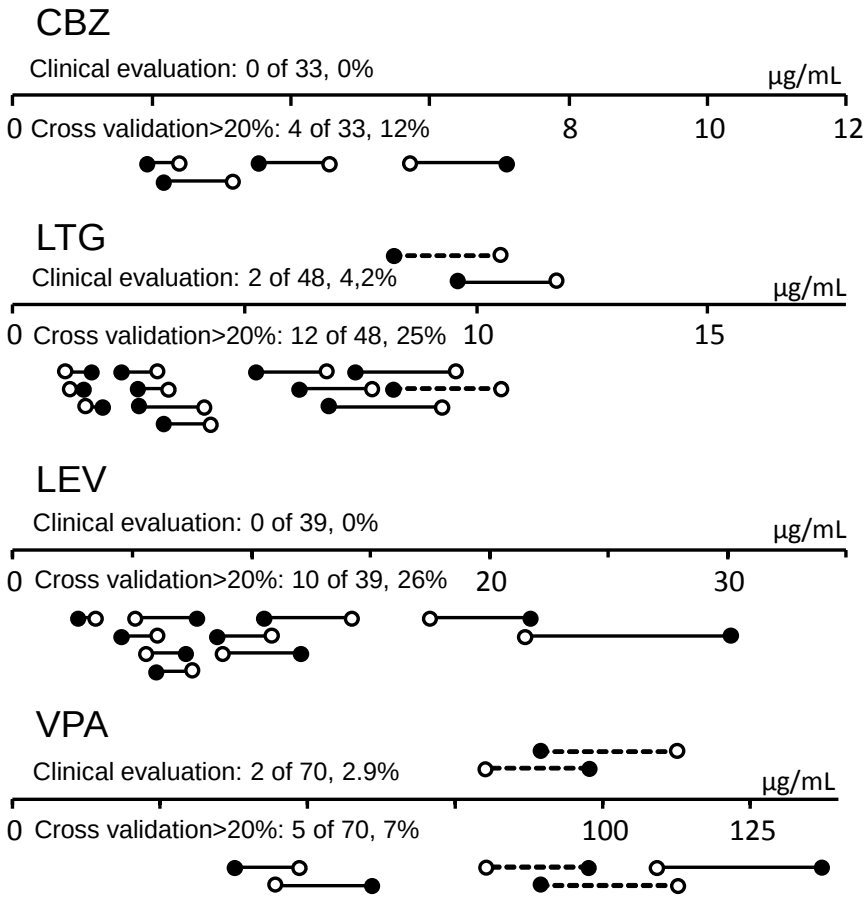


Figure 11. Samples failing criteria of cross validation and clinical evaluation of plasma and DBS samples (total of n=190 comparisons). Sample pairs that fail criteria for clinical evaluation are shown above the scale and sample pairs that fail cross validation criteria (more than 20% deviation from mean) are shown below the scale. Plasma results are filled circles and DBS results are hollow circles. A dotted line indicates that the same sample pair failed in both clinical evaluation and cross validation. CBZ=carbamazepine, LTG=lamotrigine, LEV=levetiracetam, VPA=valproic acid (re-printed from paper IV with permission)

The clinical evaluation concluded that self-sampling with DBS can be used as an alternative sampling strategy for CBZ, LTG and VPA for children with epilepsy. LEV had a few deviations over the whole concentration range and more samples are needed to evaluate if these differences arose randomly. The recommendation is to use DBS concentrations for

LEV only for compliance queries and in those patients where a traditional venous sample is not an option. The therapeutic range for LEV suggested in the literature is variating[26,159,160]. At the TDM laboratory, half of the concentrations are between 8.4–22 µg/mL. Large inter-individual differences are seen for LEV and one contributing factor for variation may be due to different concentrations in plasma if blood has not been promptly centrifuged due to in situ metabolism[156].

In general, many DBS methods have been developed and validated but results from bridging studies with patient samples have rarely been reported. It is important to perform clinical validations on capillary patient samples to ensure that the method can be used with patients.

There is a scarcity of DBS methods that have been developed and implemented in routine laboratories[161]. Other clinical evaluations or validations described in the literature are not including clinical assessment. Beyond using cross-validation limits from EMA or other organizations we have also put the results in a clinical context and evaluated the differences between the methods in relation to TDM in the individual patient.

6.3 DBS AS A SELF-COLLECTION METHOD FOR PATIENTS

Patient perspective

6.3.1 Comparison of sample quality, nurses versus guardians

In paper IV we compared two groups, nurses (n=25) and guardians (n=86), and we could see no difference in sample quality between the groups as the null hypothesis could not be rejected by Fisher´s exact test. From the DBS samples obtained by the guardians in the hospital, 2.3% were rejected due to sub-optimal quality compared to 8% in the nurse group.

The result implies that this group of guardians can collect a DBS sample from their children with acceptable quality, even though many of these children had concomitant diagnoses that made the collection difficult. In other studies where samples from children were collected at home and sent by mail to the laboratory, a much higher rate of failed samplings were reported[18,95]. Collecting DBS samples at home may meet its own challenges. It remains to be proven, if the guardians in this group can also provide acceptable samples when collected in their homes without a nurse advising them. The video instruction and the thorough training these guardians received in combination with their motivation to perform correctly, were identified as contributing factors for the successful samplings, paper V. Others have referred to improved instruction as a way to increase the rate of successful DBS samplings[18,95,162].

In paper IV and V, we prepared for self-sampling at home by developing effective self-instructive materials for guardians and children with epilepsy[1]. Results showed that guardians to children with epilepsy can collect DBS samples of acceptable quality and the aim to offer DBS collection at home instead of traditional collection at the hospital is a feasible alternative.

6.3.2 Benefits of DBS collection at home

No earlier studies have investigated the guardian´s perspective on DBS self-sampling at home. Some studies have collected data from questionnaires where the guardians and children graded the difficulty in performing self-sampling and preferability of DBS home sampling versus venous sampling at the hospital[18,95,163]. By using mixed-methods in paper V, we aimed to fill the gap of missing knowledge, where individual experiences could help to find knowledge on why or how the sampling can be successful.

Results from the questionnaire (paper V) suggested that 80% in this group of children and their guardians found DBS collection at home desirable after performing the first self-collection. The results from the questionnaire were in general positive as the participants were positively predisposed to the idea of self-sampling at home. On the other hand, it would have been difficult to investigate experiences and thoughts in a group with a low interest in performing the self-sampling at home. The participants can be seen as a subpopulation from the group of guardians and children who are interested in future home sampling. The response rate was high, out of 72 possible answers, only 8 were missing.

In the interviews, reasons for DBS collection that the guardians emphasized as important to make everyday life easier were identified. Self-collection of DBS at home, means reduced number of hospital visits and this contributes to less stress for both children and guardians. Economic factors, both on a private but also on a societal level, were another main reason. Reduced stress for the children, more flexibility for sampling and being in a relaxed home environment were identified as important factors. A population-based study on health-related quality of life in active childhood epilepsy found that anxiety, polytherapy with several AEDs and difficulties with school attendance were associated with reduced life quality[164]. Other studies point out that children with epilepsy and concomitant diagnosis and their guardians is a vulnerable group that have needs like home based care, increased autonomy and help to decrease anxieties, both for children and guardians[165–167]. In paper V, results suggest that DBS collection for TDM at home can assist in satisfying some of the needs identified in these studies. In the study, paper V, many children with neurobehavioral and cognitive impairments were included.

Another advantage brought up regarding DBS collection was the possibility to perform repeated samplings. Children with several AEDs and problems with seizures, need particularly frequent contact with health care institutions and effective TDM (more frequent sampling) might help to find the correct individual dose when epilepsy treatment is problematic. Guardians to children with epilepsy reported lack of continuity of care as problematic in one study[167].

In conclusion, possibility of DBS collection at home has been shown to be very desirable in this group of children with epilepsy, and guardians identified many advantages coupled to DBS collection at home that can reduce stress and burdens. These findings support that for

this group of patients, there is a requirement for alternative sampling that can be performed at home.

6.3.3 Contributing factors for successful DBS self-sampling

In paper V and Table 3, we identified important factors for successful self-sampling of DBS. We found that it was easier to collect a DBS sample if guardians and children with epilepsy feel confident and comfortable in the sampling situation. Identified factors for contributing to this were; prepared guardians, instructive training material (pedagogic and in diverse formats), the possibility to communicate without conflicts or fears, positive and pragmatic attitude and willingness to take on the new role as performer. Fulfilling these criteria decrease the risk of encountering problems in the sampling situation.

The knowledge and information generated as a result of the mixed-method study in paper V, can assist when creating education material as well as preparing nurses in their roles as future educators in DBS sampling[168].

Although video instruction can be effective, especially when learning practical procedures[102], we learned that providing information concerning the emotional aspects of self-sampling is often relegated. Questions related to feelings that may overwhelm the child or the guardian in the sampling situation remain unanswered The instruction videos or information by nurses can be improved by preparing the guardians not only in a practical manner but also regarding what feelings may arise in different situations.

In paper V, results showed that a comfortable environment built on trust was very important for the families involved, especially with children having concomitant diagnoses, where routines and a well-known environment affect their wellbeing. The training with a combined video instruction and a nurse they knew and were already familiar with was helpful in creating a trustful and calm situation for learning. Even though blood sampling might be less difficult in the future, because of improvements of sampling devices, the mixed-method study suggested that the practical training, with a nurse including support and feedback, during the first training occasion is important. With this thorough instruction the guardians can feel comfortable when later performing self-sampling at home.

The mixed-method design and especially the qualitative method with interview data provided complementary information that was missing from earlier studies on DBS self-sampling. The findings that self-sampling at home was desirable and that a majority could perform it without problems have also been reported by others[18,95], but how individual guardians felt and how children reacted were new interesting aspects that are important to consider when optimizing education and information in the future. These important findings could have gone unnoticed without a qualitative study. It is also valuable to identify unsuccessful samplings as these are not cost-effective and can have considerable impact from a societal perspective[16].

The aim to develop self-instructive material and an effective training for collection of DBS-samples has been achieved. Knowledge on how to improve the training to be adapted to this specific patient group has increased the possibility to collect successful DBS sampling in the future.

Societal perspective

Patient participation in health care is a societal aim that can improve health outcome and reduce costs. Cost-effectiveness has been studied and is also an important aspect of DBS sampling. In a recent study, self-sampling with DBS was shown to reduce costs up to approximately 2.5 times compared to traditional sampling by a professional at a hospital[16]. The savings in costs were mainly seen in loss of productivity for guardians and travel costs. The frequency of sampling affects the overall cost effectiveness, but DBS-collection was already cheaper after two samplings, even when a nurse had been designated for training of the sampling[16].

Positive examples of improved health care in remote areas within Sweden have been reported[169,170] and these examples can be used to exemplify how TDM in combination with DBS collection can provide better patient care. The research performed during this thesis is essential also from a societal perspective since it can help patients to be involved in their treatment, be more autonomous and reduce costs. Even if costs can be reduced, it is of importance to underline that the patient is in focus and always has the possibility to choose. For some patients, sampling at a clinic or hospital is more convenient than self-sampling at home. The choice to perform self-sampling at home must not exclude anyone, i.e. instruction videos, practical training or other instruction material must be understandable and accessible for all patients.

7 CONCLUSIONS

In this thesis, DBS as a possible matrix for AEDs in children with epilepsy was investigated. The self-sampling process was studied to be able to prepare for DBS self-sampling at home for this patient group. The findings can be valuable for researchers, professionals in laboratory medicine, nurses, clinicians, and children with epilepsy and their guardians as well as other patient groups depending on TDM.

- Simple and rapid LC-MS/MS methods with DBS as matrix for use in a routine laboratory were developed and validated for four commonly used AEDs.

- DBS collection on Whatman 903 filterpaper can be applied if the patient samples are within Hct ranges of 0.35–0.50 L/L and blood drop volumes between 15–50 µL.

- Analytes were stable on Whatman 903 filter paper up to one year in ambient temperature, can tolerate extreme temperatures and can be transported with normal mail service in envelopes with a low risk of bias on the measured concentrations.

- Based on comparison studies from 190 sample pairs from collected capillary DBS and plasma the bioanalytical cross-validation was accepted for the four AEDs, although including a few large deviations for LEV and LTG.

- Cinical evaluation found risk of generating a different TDM recommendation for DBS and plasma concentrations in only 4 out of 190 comparisons.

- DBS samples can be collected through self-sampling by the guardians or older children and TDM decisions for CBZ, LTG and VPA can be made in a safe manner.

- LEV collected as DBS can be an option for compliance queries.

- To choose DBS sampling at home as an alternative of plasma concentrations drawn at a hospital can be of value to guardians and children with epilepsy and concomitant diagnoses.

- Guardians of children with epilepsy are willing and able to collect DBS samples of acceptable quality.

- Stress factors in everyday life can be reduced if the possibility of self-sampling at home can be offered. Time and money can be saved for individuals as well as for society.

8 FUTURE PERSPECTIVES

8.1 USE OF DBS AND SELF-SAMPLING FOR TDM

As pointed out earlier in section 6.2.4, very few methods for TDM have been implemented using DBS as an alternative matrix in routine laboratories. The most suitable drugs for implementation of DBS collection in the routine laboratory are perhaps immunosuppressive drugs, where the patients are required to collect samples frequently during a long period of time. Since routine analysis of immunosuppressant drugs are done in whole blood, it is more straight forward to create DBS methods and compare results, than drugs analyzed in plasma or serum.
A disadvantage that is often mentioned is that the transplanted patients need to be monitored for other endogenous biomarkers, and as long as this is not possible with DBS, the patient still has to collect a venous blood sample. As a result, the benefits with home sampling can be realized when only drug concentrations are needed. On the other hand, many DBS methods for biomarkers and large molecules have been developed[2] and it should be possible to use one DBS collection for many types of analyses. A DBS sample, with up to 5 blood spots could be separated and used for analysis of for example, creatinine, glucose and transaminases at the clinical chemistry laboratory and for TDM at the clinical pharmacology laboratory. This approach can be of interest for patient groups that are routinely utilizing blood tests for monitoring[171].

Other TDM groups that can be of interest for self-sampling at home in the future are drugs with anticoagulant effect, NOAKs like apixaban, dabigatran, edoxaban and rivaroxaban. Monitoring of these drugs might be important to increase effectiveness and decrease adverse effects and adherence[172]. Different endogenous measurements also have to be monitored in combination for these patients and a DBS test with several spots for analysis of different laboratory tests would be practical[173].

8.2 DBS AND AUTOMATION

There are several commercially available sample work up automations for DBS[161]. The main advantages with an automation for DBS in TDM as an alternative sampling method, would be the sample traceability and to decrease the risk of manual mistakes. To increase the throughput and decrease turnaround time may not be the most important for DBS that were collected in a home environment and sent by mail to the laboratory. This process takes several days and therefore the primary aims for these samples are not fast analysis.

The automated systems are built on flow through desorption or on-line horizontally surface sealed extraction. In some of the methods internal standard can be sprayed onto the filter paper before extraction. Automatic systems for producing calibrators and QCs have also been developed and may be interesting for laboratories that handle many DBS samples[174].

8.3 VOLUMETRIC DBS

In the last five years, there has been development of blood sample collection systems with separation of a defined volume from the deposited blood drop. The analysis of the entire spot instead of a sub-punch also eliminates the Hct bias[155]. Such a volume defined, whole spot collection device for DBS has been developed[62,175] and used for parallel collection with Whatman 903, in smaller projects at the clinical pharmacological laboratory.

These developments are promising for the future since the known bias can be decreased. Most of these devices are designed to be easier for the patients to use in the self-collection situation. A disadvantage is that the cost of such a device might increase the costs for health care in comparison with the conventional filter paper and is consequently not a first choice for development countries in need of TDM. On the other hand, total savings in health care are to be expected (see section 6.3.3) if self-sampling at home can be used and the new developed devices are cost effective compared to perform the sampling at a clinic or a hospital.

8.4 OTHER MATRICES FOR SELF-SAMPLING AT HOME

For TDM and AEDs there is also a possibility of using other matrices for home sampling. Studies on AEDs and saliva have shown promising results for some AEDs[176]. The saliva sampling, however, include many uncontrollable factors and the sample can easily get contaminated. Methods for collection and automated separation of plasma on the filter paper (dried plasma spots) has been developed and is of interest in TDM[61,177–179]. This alternative might be favorable for drugs such as VPA that are mostly abundant in the plasma fraction because the extracts will probably be more comparable with routine plasma samples. If free fraction of the drug can be separated from the plasma fraction directly to the filter paper, it would be an interesting alternative for some drugs. Exhaled breath is another possible alternative that has not yet been thoroughly investigated for TDM of drugs[180].

8.5 DBS COLLECTION BENEFITS FOR TDM

DBS self-collection can facilitate larger studies related to TDM and research infrastructure becomes easier when repeated sampling can be done at home at different time-points. DBS collection can also be used for pharmacokinetic studies to improve TDM and develop better medicines for children[93,181]. Adherence and self-monitoring are other possible areas where DBS collection can be of value for TDM[182]. Field studies in countries where for example HIV and malaria medicines are most frequently used is needed. DBS can be a good alternative to venous sampling in studies to increase knowledge of pharmacokinetics as well as for compliance queries.

Large scale projects for antibiotics and tuberculosis drugs are future potential areas where DBS sampling can be valuable for research projects at the clinical pharmacology laboratory[183].

8.6 PATIENT ROLE IN THE FUTURE

There is a trend of increased patient participation and engagement in health care, such as the availability of kits for home testing, possibility to meet a doctor via the internet, order laboratory tests and see results from laboratory analysis in the electronic journal[184]. These relatively new ways to communicate and interact with the health care system have many advantages for patients living far away from hospitals or for patients with difficulties of transportation[169,170]. The new role of the patient also affects nurses, clinicians and other health care practitioners who will have to put more effort into the education and information of patients in the future. As a result of this, programs for instructions and information of patients are interesting areas for future research.

Future research projects would be to validate new sampling devices in the patient group and evaluate which of the new sampling devices are the most patient-friendly. Another possibility would be to collaborate with the companies that develop these devices and prepare automatic registration of sampling time. This would increase the effectiveness of TDM sampling, which is very much dependent on collection time. Feed-back to the patients when the sample arrives at the laboratory is another improvement that patients might ask for.

If self-sampling and analysis of DBS can be made safely, it is a way to meet the patient needs in a modern society and it can help shift the balance in an overburdened health care system.

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